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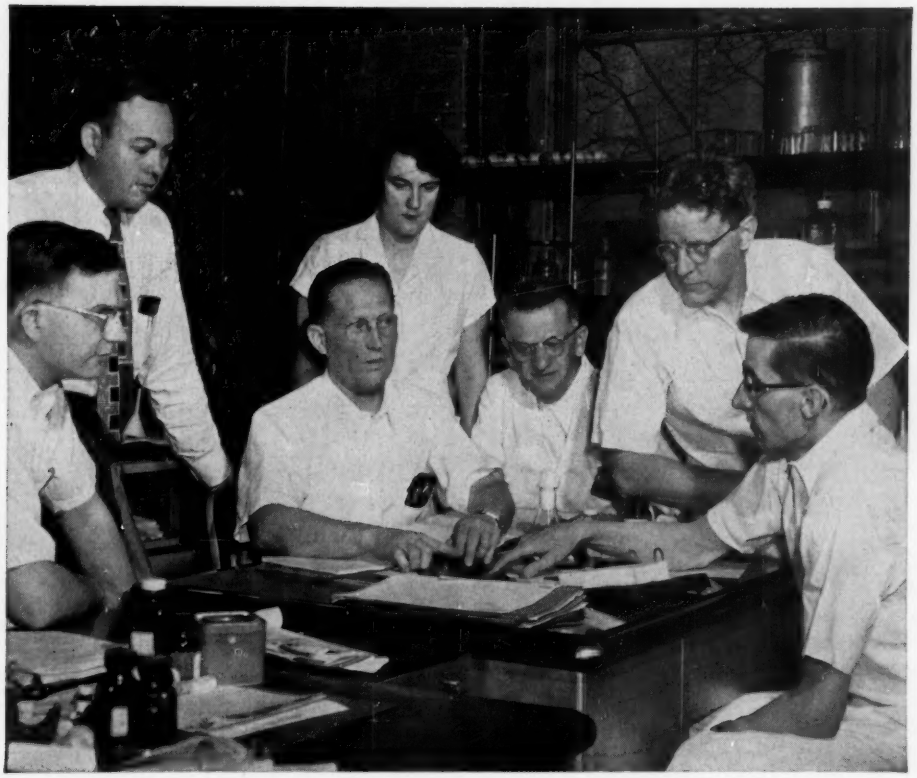

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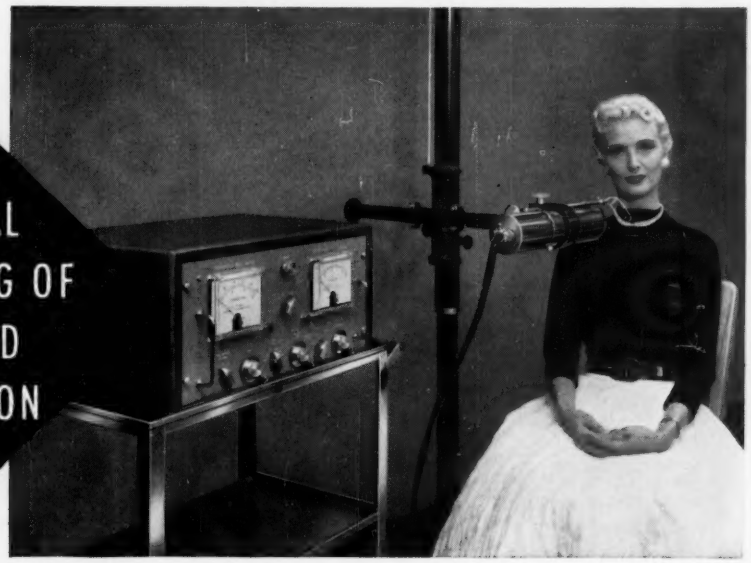

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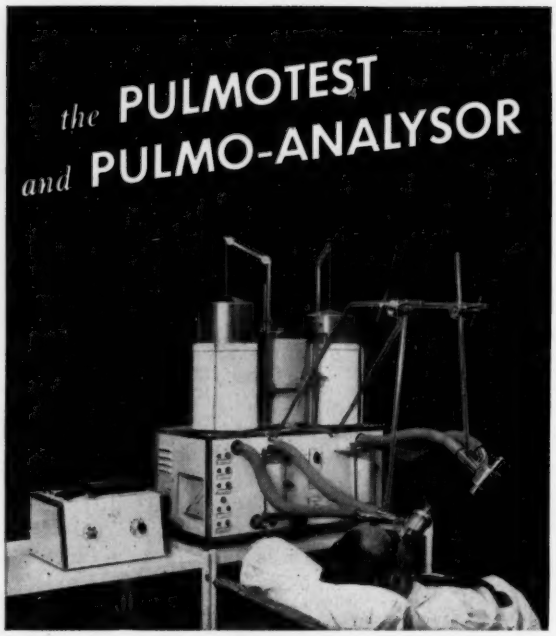

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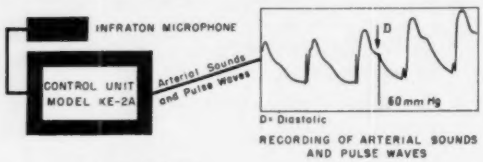

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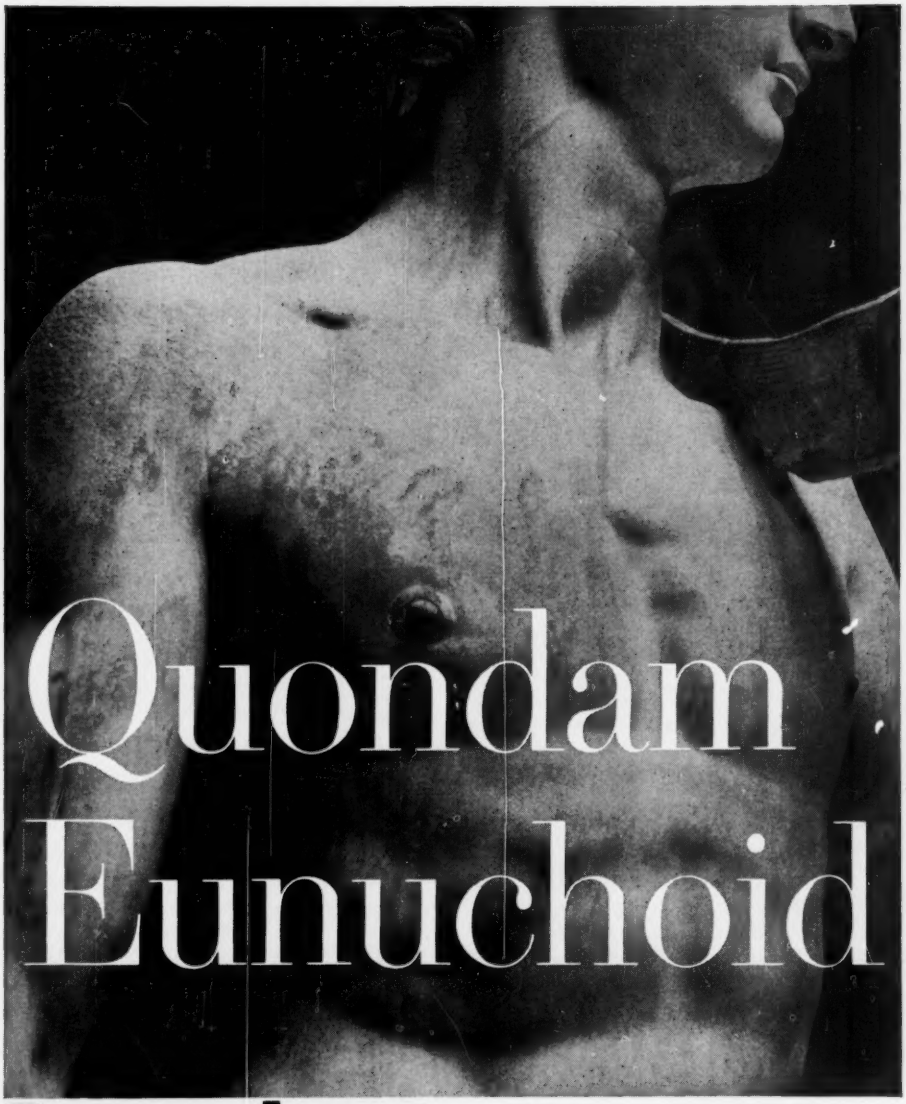

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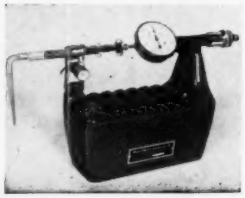

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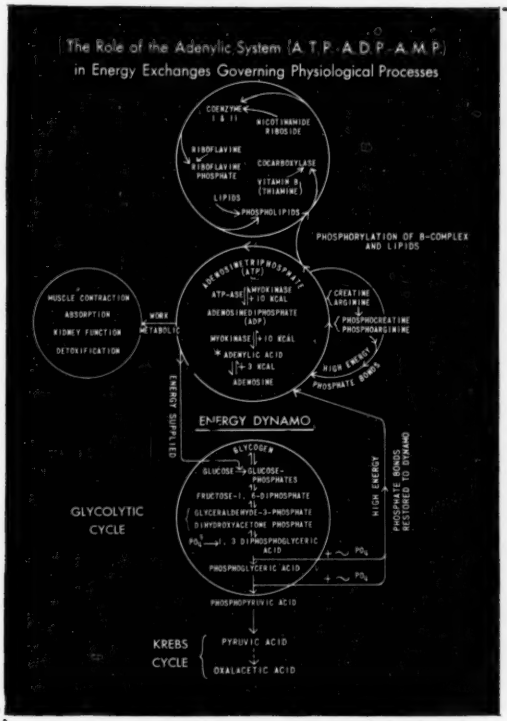

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*The American Foundation: Medical Research: A Midcentury Survey, Boston, Little, Brown and Company, 1955, vol. 1, p. XXXI.

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STUDIES ON PLATELETS. XIV. HUMAN PLATELETS AS SOURCE OF ANTIFIBRINOLYSIN [1]

By MARIO STEFANINI [2] AND IRENE S. MURPHY

(From the Joseph Stanton Memorial Research Laboratory, Saint Elizabeth's Hospital, and the Department of Medicine, Tufts University Medical School, Boston, Mass.)

(Submitted for publication October 6, 1955; accepted December 5, 1955)

Occurrence of increased plasma and serum fibrinolytic activity in patients with acute leukemia and severe thrombocytopenia (1–4) has been recently reported. It would seem that at least two mechanisms may cause this phenomenon. It is possible that profibrinolysin be activated to fibrinolysin by kinases of bacterial origin or derived from leukemic cells. It is also conceivable that thrombocytopenia may induce deficiency of circulating antifibrinolysin. The last hypothesis is supported by the findings of Johnson and Schneider (5) who found bovine platelets to contain twice as much antifibrinolytic activity as bovine plasma. Experiments to be presented in this paper discuss human platelets as source of antifibrinolytic activity and the possible role of fibrinolysis in the pathogenesis of thrombocytopenic bleeding.

MATERIALS AND METHODS

Determination of antifibrinolysin was carried out by the technic described below. The following reagents were prepared: (a) A solution of Loomis' bovine fibrinolysin,[3] containing 5 mg. per ml. in veronal buffer (6). This preparation of fibrinolysin had activity of 0.33 u. per mg.; (b) various dilutions in veronal buffer of serum or plasma under study. Determinations were made within two weeks of collection, on specimens which had been separated from the clot within one hour of venesection and kept at − 20°C. until used. Since there is evidence that some factor or factors in the fibrinolytic system are extremely labile, blood was also collected in the cold and serum separated by rapid centrifugation in the cold (4°C.) and used immediately. Results were essentially identical to those obtained with stored, frozen samples; (c) solution of bovine fibrinogen in veronal buffer, containing 300 mg. per 100 ml.; (d) solution of purified bovine thrombin in saline solution, containing 100 units per ml.

Two-tenths ml. of fibrinolysin solution and 0.2 ml. of each serum or plasma dilution were transferred to chemically clean 10 × 100 mm. serology tubes and mixture incubated at 37°C. for 30 minutes. At the end of incubation, 0.3 ml. of fibrinogen solution and 0.1 ml. of thrombin solution were added in rapid succession. A clot formed within a few seconds. A stop watch was started on the addition of thrombin to the first tube and all tubes observed at frequent intervals. The end point (*clot lysis time*) was the time at which clot had completely dissolved. A test tube where veronal buffer was substituted for serum or plasma was taken as *control*.

Platelets were counted by a modification of the direct method previously described (7) using 1 per cent solution of EDTA–Na_2 in 0.7 per cent NaCl as diluting fluid and phase contrast microscope. Technics for the determination of capillary fragility, bleeding time, prothrombin utilization during clotting (prothrombin consumption) were as previously described (4). Platelet-rich and platelet-poor plasma and suspension of washed platelets in saline were also prepared as previously described (8). Sodium citrate 0.02 M (1/10 volume of 0.2 M solution) was used as anticoagulant, since preliminary experiments disclosed that it would inhibit antifibrinolytic activity to a lesser degree than sodium oxalate or EDTA–Na_2. Silicone-coated syringes and Arquad 2-C coated needles were used for collection of blood throughout all experiments.

RESULTS

(a) *Plasma and serum antifibrinolytic activity in healthy subjects and patients with various conditions*

Antifibrinolytic activity of plasma and serum was determined in 8 patients with thrombocytosis (5 with chronic bleeding and 3 with severe hypochromic anemia); 15 patients with severe thrombocytopenia (9 with idiopathic thrombocytopenic purpura and 6 with acute leukemia); 7 patients with thrombocytoasthenia (4 of the congenital va-

[1] Supported by grant-in-aid from the American Heart Association and, in part, by grant H-2131 from the National Institutes of Health. Presented in part at the fall meeting of the American Physiological Society, Medford, Massachusetts.

[2] Established Investigator, American Heart Association.

[3] We wish to thank Dr. Eugene C. Loomis, Parke & Davis Company, Detroit, Michigan for generous supply of purified bovine fibrinolysin.

riety and 3 of the acquired variety due to chronic granulocytic leukemia). In chronic granulocytic leukemia, platelets, as in the cases studied here, may appear large and bizarre, while isolated or multiple abnormalities of platelet function may be found (poor clot-retraction, prolonged bleeding time, increased capillary fragility, poor prothrombin utilization during clotting [4]). Analytical results are presented in Table I, as those found in serum. Values for plasma were essentially identical. Antifibrinolytic activity was evidently higher in patients with increased number of platelets and in healthy subjects than in patients with quantitative (idiopathic thrombocytopenic purpura, acute leukemia) or qualitative (thrombocytoasthenia) platelet deficiency.

TABLE I

Antifibrinolytic activity of serum from healthy subjects and patients with various diseases *

	Platelet count *per cu. mm.*	Bleeding time *minutes*	Tourniquet test petechiae No.	Clot retraction %	Prothrombin utilization %	Clot lysis time, *minutes* Dilution of serum 1/10	1/20	1/30	1/40	Control
1. Normal	275,000	3½	2	51	97	58	46	35	26	13
2. Normal	224,500	2	3	43	94	57	40	31	28	14
3. Normal	282,500	5½	0	47	87	55	41	34	29	12
4. Normal	279,100	4	1	52	92	58	47	37	29	14
5. Normal	239,700	2½	5	57	89	64	44	39	35	11
6. Normal	274,000	3	0	63	74	69	54	46	40	12
7. Normal	205,150	4½	4	50	79	63	52	35	27	13
8. Normal	271,350	5½	1	42	67	67	55	32	25	12
9. Normal	237,000	3	0	61	82	57	43	39	28	14
10. Normal	301,100	2½	7	45	95	58	48	38	32	14
11. Chronic bleeding	421,000	2	0	72	100	74	62	51	35	11
12. Chronic bleeding	372,500	½	0	59	82	83	68	54	37	13
13. Chronic bleeding	402,050	2	2	69	94	75	64	58	36	14
14. Chronic bleeding	393,180	½	0	73	94	71	63	57	37	15
15. Chronic bleeding	600,000	1	0	50	100	77	62	58	40	12
16. Iron deficiency anemia	420,000	2	0	54	97	87	70	60	39	14
17. Iron deficiency anemia	372,500	3	1	47	92	89	75	63	46	12
18. Iron deficiency anemia	379,000	2½	0	71	89	94	73	64	44	14
19. Idiopathic thrombocytopenic purpura	21,000	>20	70	3	4	44	28	25	22	12
20. Idiopathic thrombocytopenic purpura	27,000	>20	68	14	0	47	29	27	19	11
21. Idiopathic thrombocytopenic purpura	41,000	18	100	7	17	37	34	26	24	13
22. Idiopathic thrombocytopenic purpura	37,000	>20	100	2	5	39	27	23	17	14
23. Idiopathic thrombocytopenic purpura	<10,000	>20	100	0	9	42	35	23	21	12
24. Idiopathic thrombocytopenic purpura	15,000	>20	74	5	4	43	34	28	19	11
25. Idiopathic thrombocytopenic purpura	25,000	17	50	0	7	36	30	22	18	12
26. Idiopathic thrombocytopenic purpura	40,000	16	35	15	8	51	37	31	24	14
27. Idiopathic thrombocytopenic purpura	55,500	14	47	9	0	50	39	30	26	11
28. Ac. lymphocytic leukemia	<10,000	>20	60	0	4	29	25	18	13	11
29. Ac. myelogenous leukemia	35,000	14	54	20	9	28	27	22	18	12
30. Ac. histiocytic leukemia	31,000	>20	64	0	7	36	29	19	16	12
31. Ac. mono-myelocytic leukemia	32,000	19	57	11	6	27	22	18	17	14
32. Ac. lymphocytic leukemia	<10,000	>20	72	7	5	28	24	17	15	13
33. Ac. monocytic leukemia	47,500	12	>100	0	3	24	20	17	15	14
34. Chr. myelogenous leukemia	1,050,000	*15*	4	52	*21*	42	30	25	22	14
35. Chr. myelogenous leukemia	755,000	*3*	*32*	*10*	*12*	44	34	27	22	13
36. Chr. myelogenous leukemia	643,000	*7*	10	47	67	40	29	24	20	11
37. Thrombocytoasthenia	275,000	*11*	7	*9*	*10*	35	33	29	24	14
38. Thrombocytoasthenia	301,500	3	10	*6*	72	35	32	27	23	10
39. Thrombocytoasthenia	226,750	*15*	15	49	79	33	25	21	20	12
40. Thrombocytoasthenia	224,500	*15*	*>100*	57	84	37	24	22	19	12

* Cases 34–40 exhibit a partial deficiency of platelet function. Results which are abnormal are in italics. It will be noted that one or more but never all platelet functions are affected.

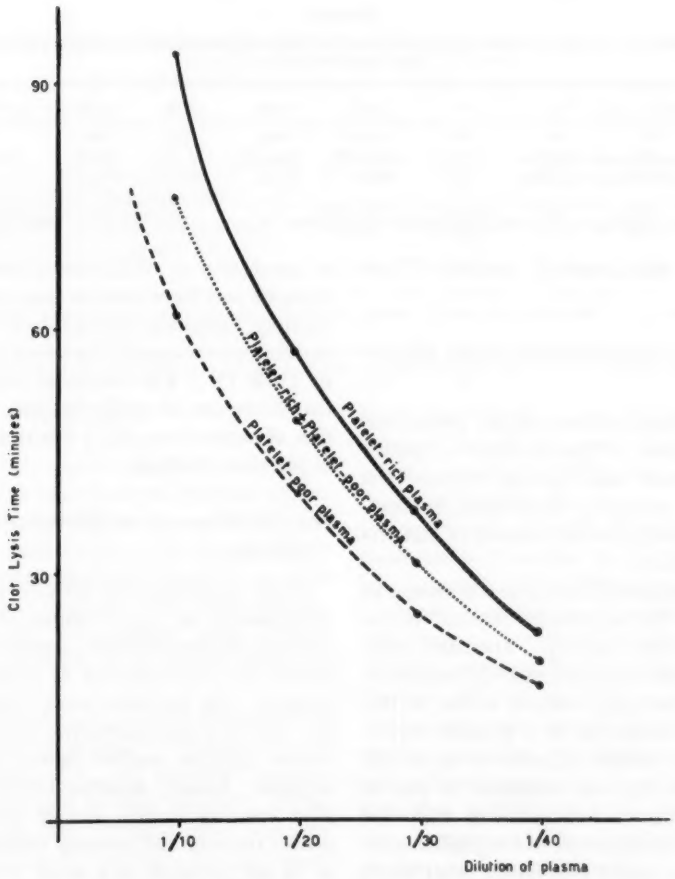

FIG. 1. ANTIFIBRINOLYTIC ACTIVITY OF PLATELET-RICH PLASMA, PLATELET-POOR PLASMA AND A MIXTURE OF EQUAL VOLUMES OF BOTH
Average results of five experiments.

(b) *Relation between number of platelets and antifibrinolytic activity of normal plasma*

Platelet-rich and platelet-poor plasma were obtained as previously described (8). Platelet-rich plasma contained an average of 300,000 platelets per cu.mm. and platelet-poor plasma of 4,000 platelets per cu.mm. A third series of samples were prepared by mixing platelet-rich and platelet-poor plasma in equal volumes. Dilutions were in veronal buffer. Results are presented in Figure 1. It is clear that platelet-rich plasma exhibited higher antifibrinolytic activity than platelet-poor plasma. Preparations containing intermediate number of platelets also exhibited intermediate antifibrinolytic activity.

(c) *Antifibrinolytic activity of serum obtained by recalcification of platelet-rich or platelet-poor plasma*

Two samples of blood were collected with "Silicone technic" (9) using 1/10 volume of 0.2 M sodium citrate as anticoagulant. Platelet-rich and platelet-poor plasma were then obtained and recalcified by addition of 1/10 volume 0.2 M Ca Cl_2. After incubation at 37°C. for one hour, serum was separated and tested for antifibrinolytic activity. Serum obtained from platelet-rich plasma

TABLE II

Antifibrinolytic activity of serum obtained by recalcification of platelet-rich or platelet-poor plasma (ten experiments) *

Dilution of serum	1/10	1/20	1/30	1/40	Control
	min.	*min.*	*min.*	*min.*	*min.*
Serum from platelet-rich plasma	101±12	76±10	54±8	30±5	12±3.5
Serum from platelet-poor plasma	68±7	37±5	28±4	17±4	12±3.2

* Clot lysis time—Average value and standard deviation given (figures approximated to one-minute intervals).

exhibited higher antifibrinolytic activity (Table II).

(d) *Liberation of antifibrinolysin from platelet-rich plasma*

Platelet-rich plasma was repeatedly frozen and thawed. Observation of plasma after a number of these steps showed many platelet "ghosts" but also considerable clumping of platelets, preventing actual determination of the number of platelets either intact or lyzed.

The effect of repeated freezing and thawing, as shown in Table III, apparently resulted in increased antifibrinolytic activity. The same table shows, however, that such procedure, if continued for a long time, eventually resulted in loss of this effect. This was confirmed in a parallel experiment, when the fibrinolytic activity of serum left at 20°C. for three days was compared to that of another sample thawed and refrozen daily for three days. The second sample had negligible antifibrinolytic activity, which was, on the other hand, intact in the first sample which had been kept continuously frozen.

Another technic consisted in adding to platelet-rich plasma 2/10 volume of either normal inactivated serum or of serum from a patient with idiopathic thrombocytopenic purpura exhibiting a high titer platelet agglutinin. After one hour of incubation at 37°C., many platelets appeared clumped and there were several platelet "ghosts," possibly indicating platelet lysis. Liberation of antifibrinolytic activity by such serum is shown in Table IV. The results of Table V indicated that liberation of antifibrinolysin through the action of serum was also a function of the number of platelets available.

(e) *Liberation of antifibrinolysin from washed platelets*

Good antifibrinolytic activity was found after resuspending in equal volume of saline solution without washing, platelets separated from citrated plasma by centrifugation at 3,000 r.p.m. for 15 minutes. On the other hand, Table IV indicated the very low antifibrinolytic activity of isolated human platelets washed three times with saline solution. Equally negative results were obtained after treating washed human platelets with repeated freezing and thawing either in the absence or in the presence of a small volume of normal human plasma or serum, addition of saponin (0.007 per cent), thrombin, acetone. The addition of serum from patients with idiopathic thrombocytopenic purpura was also ineffective. None of these agents, which clumped platelets heavily, were able to liberate antifibrinolysin from washed platelets.

TABLE III

The effect of repeated freezing and thawing on antifibrinolytic activity of platelet-rich plasma (ten experiments) *

Dilution of plasma	1/10	1/20	1/30	1/40	Control
	min.	*min.*	*min.*	*min.*	*min.*
Platelet-rich plasma†	87±9	58±7	38±5	21±4	12±3
Platelet-rich plasma, 3× frozen and thawed	158±20	87±11	63±7	42±4	12±2
Platelet-rich plasma, 12× frozen and thawed	34±4	25±4	18±3	14±2	12±3

* Clot lysis time—Average value and standard deviation given (figures approximated to one-minute intervals).
† Containing 300,000 platelets±20,000 per cu. mm.

TABLE IV

Liberation of antifibrinolysin from platelet-rich plasma on addition of inactivated serum from patients with idiopathic thrombocytopenic purpura *

Dilution of plasma	1/10	1/20	1/30	1/40	Control
	min.	*min.*	*min.*	*min.*	*min.*
Platelet-rich plasma†	86±8	62±7	36±6	24±3	12±4
Platelet-rich plasma + normal serum‡	101±16	79±8	48±5	31±4	13±2
Platelet-rich plasma + serum of patient with idiopathic thrombocytopenic purpura‡	153±18	98±12	73±7	53±7	11±4
Platelets in saline†	18±4	15±3	13±3	13±2	12±3
Platelets in saline + serum of patient with idiopathic thrombocytopenic purpura‡	22±4	18±5	15±4	15±4	11±3

* Clot lysis time—Average value and standard deviation given (figures approximated to one-minute intervals).
† Containing 300,000 platelets±35,000 per cu. mm.
‡ Two-tenths ml. of serum from patient with chronic idiopathic thrombocytopenic purpura (containing platelet agglutinin with titer 1/128) or normal donor added to 0.8 ml. of platelet-rich plasma or platelet suspension in saline.

DISCUSSION

The experiments reported should be interpreted with caution. The mixed use of reagents of human and bovine origin and the necessity of using anticoagulants, which inhibit to a certain degree the activity of antifibrinolysin, may have, at least in part, vitiated the findings. For these reasons, it was felt necessary to overcome some of these difficulties by a multiple experimental approach to the problem.

Even so, conclusions suggested by the preceding experiments are only tentative. Antifibrinolytic activity of plasma and serum, however, appears related to platelets and to their number. Thus, serum of patients with thrombocytosis exhibits higher antifibrinolytic activity than normal serum, and this, in turn, is more active than serum from patients with quantitative (thrombocytopenia) or qualitative (thrombocytoasthenia) platelet deficiency. It is significant to note that serum from patients with idiopathic thrombocytopenic purpura exhibits perhaps higher antifibrinolytic activity than that from patients with the secondary type of thrombocytopenia (acute leukemia or aplastic anemia), a finding for which we have no explanation at this time.

Experiments *in vitro* have also confirmed the relationship of number of platelets to antifibrinolytic activity. This is higher in platelet-rich than in platelet-poor plasma and in serum from platelet-rich than in serum from platelet-poor plasma. Yield of antifibrinolytic activity, possibly through the lytic effect of serum from patients with idiopathic thrombocytopenic purpura, is largest in plasma containing the highest number of platelets. There seems to be sufficient evidence to state that antifibrinolysin in human blood is, at least in part, related to platelets, or to platelet products or represents material bound to or carried by platelets. The experiments, however, do not answer definitely the question whether antifibrinolysin constitutes an intimate component of platelets or is simply carried by them. The enhancement of antifibrinolysin activity in platelet-rich human plasma by repeated freezing and thaw-

TABLE V

Liberation of antifibrinolysin from platelet-rich plasma on addition of inactivated serum from patient with idiopathic thrombocytopenic purpura (six experiments) *

Dilution of plasma		1/10	1/20	1/30	1/40	Control
		min.	*min.*	*min.*	*min.*	*min.*
Platelet number	800,000/cu. mm.	102±12	72±11	43±7	27±4	13±4
	600,000/cu. mm.	89±10	60±9	41±7	24±4	13±3
	300,000/cu. mm.	78±9	54±7	34±5	20±3	13±2
	150,000/cu. mm.	72±8	49±7	27±6	19±4	13±4
	20,000/cu. mm.	61±9	43±6	24±5	15±2	13±3

Two-tenths ml. of serum from patient with chronic idiopathic thrombocytopenic purpura (containing platelet agglutinin with titer 1/128) added to 0.8 ml. of platelet-rich plasma.

* Clot lysis time—Average value and standard deviation given (figures approximated to one-minute intervals).

ing and by addition of serum from patients with idiopathic thrombocytopenic purpura seems to indicate that antifibrinolytic activity is liberated as platelets are destroyed. On the other hand, no antifibrinolysis can be obtained from human platelets after repeated washing. It is still present, however, if platelets are only separated from plasma by single centrifugation and resuspended in saline solution. This last experiment could be interpreted as evidence that antifibrinolysin may be washed away from platelets.

The discrepancy between the various experimental results may best be explained by a number of considerations. It appears, first of all, difficult to lyze platelets which have been washed free of plasma or serum. Also, antifibrinolysin might have been lost during the process of separation and washing of platelets. It appears unlikely that a factor or factors present in serum or plasma may be necessary for the release of antifibrinolysin from platelets since addition of serum or plasma to washed human platelets fails to increase antifibrinolytic activity of the mixture, beyond the antifibrinolytic activity supplied by serum or plasma themselves. Whether platelets contain or carry antifibrinolysin is not clearly demonstrated by these experiments. These studies, nevertheless, raise the question whether platelets may not only contain but also carry factors with important biological function and other circulating chemicals.

Recent work has shown the presence in platelets of a number of factors which are specifically active in various phases of the hemostatic process (10). It appears that at least some of them may represent agents carried by or absorbed into the platelets rather than intimate constituents of platelets. Thus it is thought by some investigators (11) that serotonin, adrenalin (11), perhaps histamine are possibly absorbed on platelets. Administered Dextran may coat the platelet surface (12). None of the experimental results presented contradict the conclusion that antifibrinolysin also may be carried by or absorbed into platelets.

Finally, the observations reported here may have clinical significance. As mentioned in the introduction, increased fibrinolysis may be encountered in patients with severe secondary thrombocytopenia (aplastic anemia, acute leukemia) and be accompanied by severe purpura. We have recently encountered one such case. Transfusion of platelets as platelet-rich plasma, but not of separated, washed human platelets induced considerable decrease in the bleeding manifestations which was accompanied by reduction of fibrinolysis and increased antifibrinolytic activity of serum (Table VI).

SUMMARY

1. Antifibrinolytic activity of serum is higher in patients with thrombocytosis than in patients with quantitative or qualitative platelet deficiency. *In vitro,* antifibrinolytic activity of plasma is directly proportional to the number of platelets available and, in platelet-rich plasma, is enhanced by procedures leading to lysis of platelets (repeated freezing and thawing, addition of platelet-lytic serum). No antifibrinolysin, however, may be obtained from human platelets washed repeatedly. Addition of plasma or serum to washed platelets also fails to release antifibrinolysin. Thus, whether platelets contain, carry or absorb antifibrinolysin requires further investigation. The available evi-

TABLE VI

Effect of platelet transfusion (platelet-rich plasma) on serum antifibrinolytic activity in a patient with acute histiocytic leukemia *

Dilution of serum		1/10	1/20	1/30	1/40	Control	Platelet count (Direct method)/ cu.mm.
		Clot lysis time, *minutes*					
Before transfusion		32	27	21	17	13	12,000
After transfusion	5 min.	60	52	43	30	13	125,000
	2 hrs.	43	40	38	27	13	105,000
	24 hrs.	39	38	31	25	13	65,000
	48 hrs.	39	29	24	22	13	39,000
	72 hrs.	37	29	24	18	13	18,000

* Patient, 29 year-old male, weight 110 pounds, received 250 ml. of platelet-rich polycythemic plasma containing 1,100,000 platelets per cu.mm.

dence seems to suggest that platelets are carriers of antifibrinolysin.

2. Elevated fibrinolytic activity may be seen in patients with severe thrombocytopenia. The finding may be explained in some instances by low blood antifibrinolytic activity, which, in such cases, may become an important pathogenetic factor of bleeding.

ACKNOWLEDGMENT

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THE INDUCTION OF GROSS MYOCARDIAL LESIONS BY A COXSACKIE (PLEURODYNIA) VIRUS AND CORTISONE [1, 2]

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Coxsackie viruses are defined by their virulence for infant mice and hamsters and their inability to induce manifest disease in adults of these species. It has been shown that the administration of cortisone to the adult mouse changes this state of intrinsic resistance to one of marked susceptibility resulting in death (1). This dramatic effect is brought about only with those strains of Group B (pleurodynia) Coxsackie virus which can multiply to some extent in the adult mouse in the absence of cortisone (2). Infection of normal adult mice with Group B viruses, types 1 and 3, results in non-fatal disease associated with pathologic changes largely confined to the pancreas (3, 4). The administration of cortisone results in prolonged viremia and persistence of virus in the lung and brain (4).

In the present investigation efforts had been made to effect a lethal adaptation of a strain of Group B, type 3 virus to the adult mouse by serial passage in the cortisone-injected host. The virus employed was recovered directly in cortisone-injected adult mice from the stool of a patient with epidemic pleurodynia, without intervening passage in infant mice. On the 11th passage, gross necrotic lesions of the myocardium were discovered. The nature and pathogenesis of these lesions are the subject of this paper.

MATERIALS AND METHODS

Virus—A virus (1302) recovered from a pleurodynia patient's stool in New Jersey in 1950 by the senior author was used in most experiments. The line of virus designated "infant mouse virus" was recovered from the same stool and passed exclusively in infant mice by intraperitoneal inoculation of whole carcass suspensions. The history of the adult mouse strain is detailed in Table I. We are indebted to Dr. Gilbert Dalldorf for his classification of this agent as a Group B, type 3 Coxsackie virus. The Conn. 5 strain of Group B, type 1 virus and another Group B, type 3 strain supplied by Dr. Dalldorf were employed in one experiment. Both strains had previously been passed only in infant mice.

Antibiotics—Viral suspensions were inoculated in 0.5-cc. volumes which included final concentrations of 10 units of penicillin and 1 mg. of streptomycin per cc. Control saline injections also included both antibiotics.

Cortisone—Cortisone was administered in the form of Cortone® (Merck) in 1 to 5-mg. dosage subcutaneously. Control mice received the suspending agent used in this preparation.

Mice—Sixteen to twenty Gm. male CFW mice were used in all experiments. Infant mice were of the same strain and were less than 48 hours old when inoculated.

Infectivity titrations—Infectivity titrations in adult mice were effected by the intraperitoneal injection of 0.5-cc. amounts of virus dilutions in groups of four mice concomitantly given 5 mg. of cortisone subcutaneously. LD_{50} was calculated in the usual manner. Mice were observed for 10 days.

Scoring of lesions—A quantitative estimation of the extent of gross myocardial necrosis was made according to the following scoring system: lesions involving 10 per cent or less of the myocardial surface, 1; 10 to 30 per cent, 2; 30 to 50 per cent, 3; > 50 per cent, 4. The cumulative score of a group of mice was determined by the formula $\frac{(n \times 1) + (n \times 2)}{\text{total } n \times 4}$ etc., when $n \times 4 =$ maximum possible score.

Preparation of immune serum—Specific immune serum against the 1302 strain was produced by intraperitoneal injection of thirty 25 to 30-Gm. adult mice with 0.5 cc. of a 10^{-3} No. 2 Coor's filtrate of suckling mouse suspension containing infective virus. After 21 days mice were bled from the heart and sera pooled.

Histologic examination—Tissues were fixed in Bouin's solution for one hour. Paraffin block sections were stained with hematoxylin and eosin.

Tissue cultures—Hela cell cultures were obtained as tubes of monolayer cultures from Microbiological Associates, Inc. Monolayer monkey kidney cultures were obtained through the courtesy of Dr. Henry Gelfand from the laboratory of Dr. John P. Fox.

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[2] A preliminary report of this work was presented before the Central Society for Clinical Research on October 30, 1953.

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RESULTS

Adaptation of 1302 virus to adult mice

In the sense of selecting or inducing mutation of a viral variant lethal for the adult mouse, adaptation has not been accomplished. However, study of Table I will demonstrate that a measure of adaptation to the cortisone-injected mouse has been effected as indicated by the greater extent of myocardial necrosis in later passages. Passage without cortisone resulted in multiplication of virus in the myocardium, but no gross myocardial disease (passages 18–21, Table I).

The occurrence of gross myocardial lesions

The first ten viral passages were effected by brain suspensions until cardiac lesions were noted, by chance, on the 11th passage. Subsequently, the 15th to 22nd passages were made with heart suspensions after myocardial lesions were consistently observed in passages 12 to 14. The grossly evident lesions are illustrated in Figure 1. Microscopic examination (Figure 3) defined these lesions as large areas of necrosis characterized by degenerating muscle fibers showing fragmentation, cytoplasmic granularity or "smudging," and frequently, increased basophilia. Many of the degenerating fibers were anuclear or had pyknotic nuclei. Fragments of nuclear debris were frequently seen scattered through the areas of necrosis. There was a mild to moderate degree of infiltration of the areas of necrosis by large and small mononuclear cells, and in many instances, polymorphonuclear leukocytes were noted scattered throughout the infiltrate.[4]

TABLE I

*Serial passage of **1302** virus in cortisone-injected adult mice* *

Passage	Virus source	Dilution†	Inoc. route	Cortisone dose	Cardiac lesions
				mg.	
1	Stool	1.0	i.c.	5	‡
2	Brain	2.3	i.p.	5	§
3	Brain	5.0	i.p.	5	§
4	Brain	2.0	i.p.	5	‡
5	Brain	1.3	i.p.	5	§
6	Brain	2.0	i.p.	5	§
7	Brain	2.0	i.p.	5	§
8	Brain	2.0	i.p.	1	§
9	Brain	2.0	i.p.	1	§
10	Brain	2.0	i.c.	1	§
11	Brain	2.0	i.p.	1	‖
12	Brain	2.0	i.p.	1	‖
13	Brain	2.0	i.p.	5	‖
14	Brain	2.3	i.p.	5	‖
15	Heart	2.0	i.p.	5	‖
16	Heart	2.0	i.p.	5	‖
17	Brain	2.0	i.p.	5	‖
18	Heart	2.0	i.p.	0	¶
19	Heart	2.0	i.p.	0	¶
20	Heart	2.0	i.p.	0	¶
21	Heart	2.0	i.p.	0	¶
22	Heart	2.0	i.p.	5	‖

* Cortisone dosage was reduced in passages 8–12 in an effort to assess the degree of adaptation of the virus. Induction of lethal disease in all animals was not effected by the lower dose, so that the original dosage regimen was resumed.

† Log of dilution.

‡ Small areas of necrosis and cellular infiltration evident on microscopy of repetitions of this passage.

§ Hearts not examined for lesions.

‖ Grossly apparent cardiac lesions.

¶ Cardiac lesions not grossly apparent.

[4] The authors are indebted to Dr. Alan Rabson for his assistance in the interpretation of tissue sections.

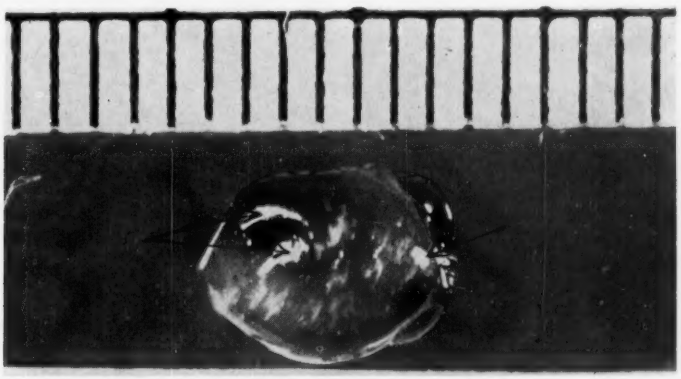

FIG. 1. GROSS LESIONS INDUCED BY VIRUS AND CORTISONE
(Scale in millimeters.) Arrows indicate high lights.

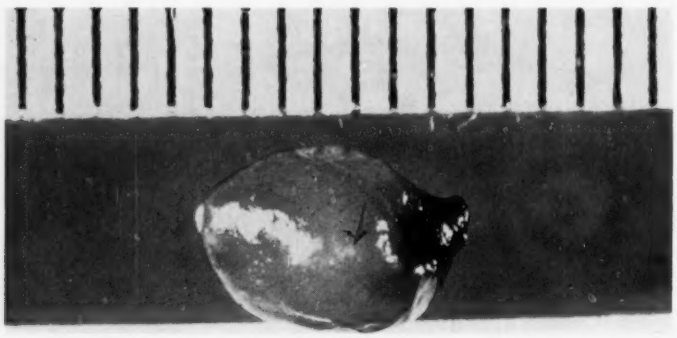

FIG. 2. GROSS LESIONS ASSOCIATED WITH CORTISONE INJECTION ALONE (ARROW)

The large white area on the left is an artifact of light reflection (high light).

Myocardial lesions in control mice injected with cortisone

The sporadic occurrence of tiny but grossly evident heart lesions in mice given cortisone alone (Figure 2) prompted section and microscopic examination of these hearts. As is demonstrated in Figure 4, the predominant lesion in grossly damaged hearts was found to be a coagulation necrosis with minimal cellular infiltration which was frequently associated with periarteritis. However, section of hearts without gross necrosis from cortisone-injected animals revealed only focal necrosis and infrequent evidence of vasculitis and valvulitis. Evidence that the cortisone lesions differ from those associated with administration of virus, or virus plus cortisone, is summarized in Table II. The sharply focal nature of the necrosis and inconstant occurrence of gross lesions together with their lack of transmissibility are arguments against their identity with Coxsackie virus-related lesions. Lesions associated with cortisone alone appeared to differ as well from previously described "spontaneous" murine carditis (5, 6). It is of interest that gross cardiac lesions were reported in 17 per cent of rats given large amounts of cortisone acetate (7). The nature of these lesions was not defined.

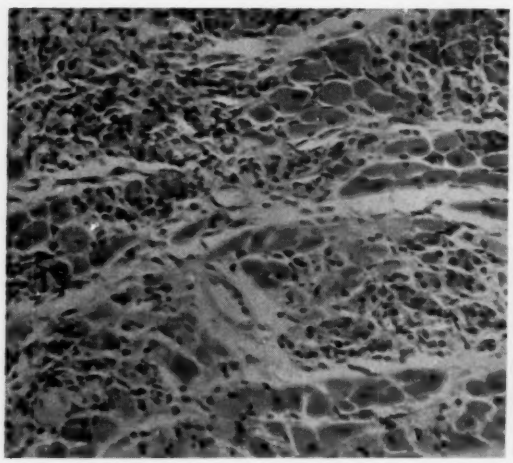

FIG. 3. MYOCARDIAL NECROSIS INDUCED BY VIRUS AND CORTISONE

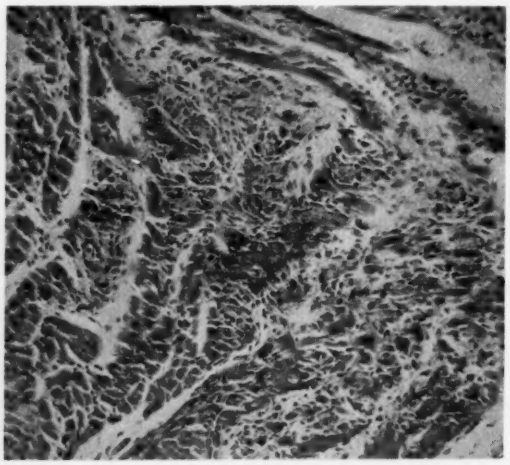

FIG. 4. FOCAL MYOCARDIAL LESION ASSOCIATED WITH CORTISONE ALONE

TABLE II

Comparison of myocardial lesions associated with virus and cortisone *

	Virus	Virus + Cortisone	Cortisone
Incidence†	25%	87%	16%
Distribution	Focal	Diffuse	Focal
Extent	+	++++	+
Cell infiltrate	++	++	+
Necrosis	+	++	++
Periarteritis	0	0	±
Valvulitis	0	0	±
Transmissibility	+	+	0
Specific neutralization	+	+	0

* Cumulative experience of six experiments in which mice were examined 6 to 12 days after inoculation.
† Based on examination of 76 mice for gross lesions.

In Figure 5, reference may be made to a normal mouse myocardium.

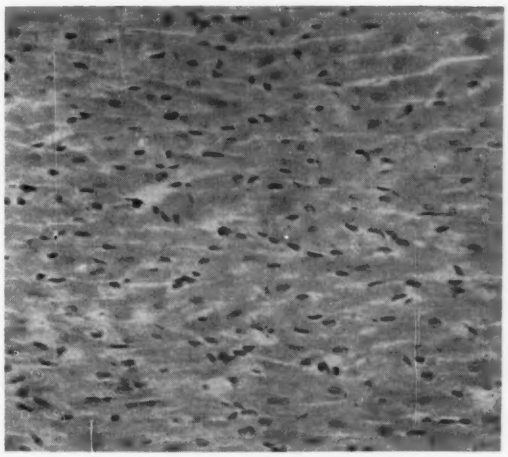

FIG. 5. NORMAL MOUSE MYOCARDIUM

The influence of cortisone administration on the induction of myocardial lesions in infected mice

In mice injected with virus alone, gross cardiac lesions were seldom observed until 9 to 12 days after inoculation, although infiltrative lesions with minimal necrosis (Table II) were occasionally noted by microscopy. The supplementary administration of cortisone at the same time or as late as three days after viral inoculation resulted in gross myocardial necrosis which became evident five days or more after initiation of infection. A typical experiment is presented in Table III. That gross necrosis is not related to virus concentration *per se* is suggested by the high titer of virus in mice without gross lesions which had received virus only although persistence of virus is suggested in mice given cortisone on the 5th day. Examination of Figure 6 reveals the lack of effect of cortisone on the rate of myocardial viral increase. This fact together with the observation that cortisone may induce necrosis when given near the peak of viral increase indicate that necrosis probably is not related to modification of viral multiplication.

Prevention of myocardial lesions with specific viral antiserum

The dependence of gross myocardial necrosis on the injection of virus is illustrated by the absence of lesions in mice which received a mixture of late passage 1302 virus and specific 1302 antiserum (Table IV).

TABLE III

The influence of cortisone administration on the induction of myocardial lesions

Group 4	Cortisone given	Hearts examined	d/t	Cardiac lesion score†	Cardiac virus inf. titer
1	none	Day 5	0/8	0 0 0 0 0 0 0 0	6.0
2	Day 1*	Day 5	3/8	3 3 3 3 3 – – –	>6.3
3	none	Day 7	0/8	1 0 0 0 0 0 0 0	>6.0
4	Day 3	Day 7	5/8	3 3 3 – – – – –	6.7
5	none	Day 9	0/8	0 0 0 0 0 0 0 0	4.5
6	Day 5	Day 9	0/8	1 1 1 1 1 0 0 0	5.7

* 1 day after injection of 10^{-3} dilution of 18th passage virus.
† 3—Lesions grossly involving 30 to 50 per cent of the myocardial surface.
1—Lesions grossly involving 10 per cent of the myocardial surface.
0—No grossly evident lesions.

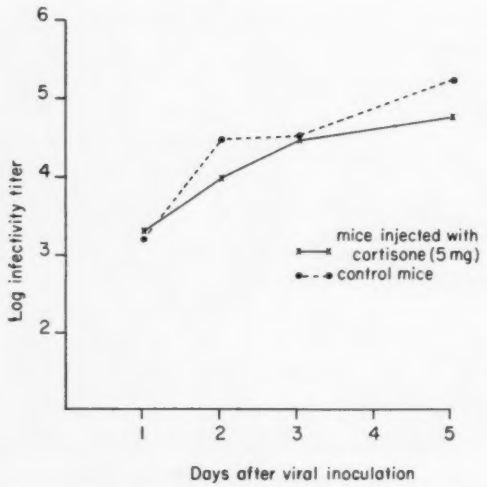

FIG. 6. COMPARATIVE INCREMENTAL CURVES OF MYOCARDIAL VIRUS IN MICE WITH AND WITHOUT CONCOMITANT CORTISONE (19TH PASSAGE)

Each point represents the titration end-point of pooled myocardial suspensions from five mice.

Myocardial lesions in infant mice

Neither gross nor microscopic lesions have been observed in paralyzed infant mice infected with either the infant mouse or adult mouse line of 1302 virus. Observations have not been extensive, but have been sufficient to establish that myocardial lesions must be considerably less common in infant mice than in cortisone-injected adults.

Multiplication of infant mouse 1302 virus in the hearts of adult mice not injected with cortisone

The capacity of 1302 virus to increase in the myocardium without previous adult mouse passage has been demonstrated by inoculation of untreated adult mice with infant mouse virus. Assay of 10 pooled hearts removed at the 4th day revealed an LD_{50} titer of 10^{-6}.

Multiplication of virus in hearts of normal adult mice on primary isolation from human feces

An experiment was designed to determine whether clinically inapparent intracardiac multiplication of virus might occur *in the absence of cortisone* on direct isolation of virus from human material in adult mice. A 10^{-3} suspension of feces (from which a virus immunologically identical with 1302 had been previously recovered) was injected into eight adult mice. Four mice received cortisone at the time of inoculation; all were dead by the fourth day. The remaining four mice were killed on the seventh day and their hearts removed for assay of virus in cortisone-injected adult mice. The infectivity titer of 10^{-4} of the cardiac homogenate demonstrated the inherent capacity of this virus to propagate in cardiac tissue in the absence of exogenous cortisone.

Biological properties of 1302 virus passed in adult mice; avirulence for human and simian tissue culture

Because of the demonstrated cytopathogenicity of Group B Coxsackie viruses in tissue culture (8–10), attempts were made to titrate adult mouse 1302 virus by this convenient method. Surprisingly, cytopathogenic effects were not noted by the seventh day in either human (Hela) or Monkey (kidney) cell cultures, despite the demonstrable cytopathogenicity of the infant mouse line of the same virus (Table V). Further study of this phenomenon is illustrated in Figure 7. Two attempts to induce tissue culture cell damage with adult mouse virus were unsuccessful on initial passage. Although multiplication of virus was not demonstrable in Hela cells, extensive increase in virus not associated with visible cytopathogenic effects occurred in monkey kidney tissue. Such virus remained adult-mouse virulent but even on second passage was relatively avirulent for tissue culture (*i.e.*, cytopathogenic in lower dilution). Even passage of adult mouse virus in infant mice

TABLE IV

Prevention of myocardial lesions with specific 1302 viral anti-serum

Mice injected with			Myocardial lesions	
Virus	Serum	Cortisone	Gross	Microscopic†
+	Normal*	+	4/4	
+	Anti-viral	+	0/4	0/2
0	0	+	0/4	
+	Saline	0	0/4	1/2
+	Saline	+	4/4	

* Pooled serum from normal mice.
† Two hearts from each group sectioned at three levels.

TABLE V

Avirulence for human and simian tissue culture of virus passed in the adult mouse

Virus passed only in:	Virus titer in: Adult mice	Monkey kidney tissue culture	Hela cell tissue culture
Adult mice	4.5	<2.0	<2.0
Infant mice	5.7	5.5	4.0

did not immediately induce tissue culture pathogenicity. In contrast, 1302 infant mouse virus showed comparable titration end points in both adult mice and monkey kidney tissue. Thus, passage in the adult mouse has resulted in the emergence of virus which differs in at least one biological property from the same isolate passed in infant mice.

Comparative incidence and degree of gross and microscopic myocardial lesions in mice infected in a single experiment with different strains of virus

Although the cumulative experience of the previously described experiments afforded clear evidence concerning the relative occurrence of cardiac lesions in cortisone and virus-injected mice, strict comparative assessment of lesion incidence could not be made from data available because of the differing observation periods of experiments (Table II). To determine more definitively the incidence and degree of cardiac necrosis, an experiment was devised in which 222 mice which averaged 19.5 Gm. in weight were inoculated on the same day. Subsequently, all

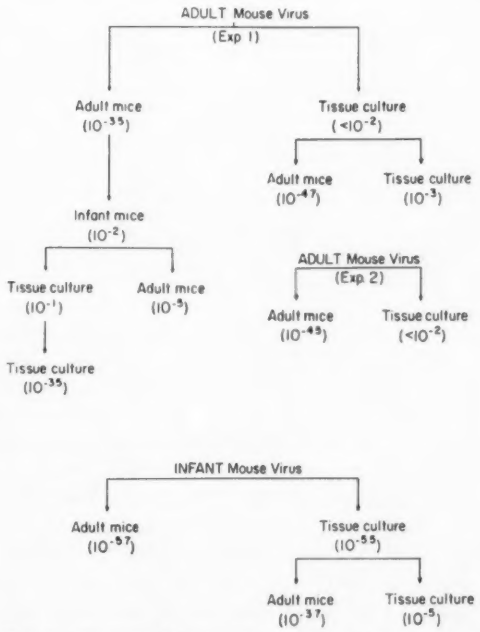

FIG. 7. REDUCED VIRULENCE OF ADULT MOUSE VIRUS FOR MONKEY KIDNEY TISSUE CULTURE

Inapparent multiplication of mouse-virulent virus. Infectivity titration end points (LD_{50}—mouse, and TCD_{50}—tissue culture) are indicated parenthetically.

TABLE VI

Comparative incidence and degree of gross myocardial lesions in mice infected in a single experiment with different strains of Group B Coxsackie viruses *

(Based on examination of 160 mice)

Virus	Strain	Amt. inoc.	Cortisone‡	Mortality§	Gross lesions‖ Incidence	(%)	Score	(%)
B-3	1302 A	2.4†	none	0/25	1/24	4.1	1/96	1.0
B-3	1302 A	2.4	+	15/46	19/31	61.3	45/124	36.2
B-3	1302 I	3.6	+	30/45	4/9	44.4	6/36	16.7
B-3	Dalldorf I	3.6	+	7/25	11/18	61.0	20/72	27.7
B-1	Conn 5 I	3.3	+	3/31	3/28	10.7	3/112	2.6
Saline	—		+	0/25	1/25	4.0	1/100	1.0
Saline	—		none	0/25	0/25	0.0	0/100	0.0

* A—Virus previously passed only in adult mice; I—Virus previously passed only in infant mice.
† Log of LD_{50}.
‡ Dose—5 mg. subcutaneously.
§ Cumulative at the 6th day. Number dead/total in group.
‖ Gross lesions: Incidence — fraction of group exhibiting lesions 1+ or greater. Score — Denominator = Number of mice examined × 4 when 4 is the maximum possible lesion score (50 per cent of myocardial surface). Numerator = Cumulative actual score of group. Percentile expression makes possible comparison of scores in groups of varying size. All lesions graded on the 6th day of infection.

TABLE VII

Incidence of microscopically evident myocardial necrosis. Microscopic examination of selected hearts from experiment detailed in Table VI*

Group	Virus	Strain	Cortisone	Necrosis		Perivascular infiltration
				Focal	Disseminated	
1	B-3	1302 A	None	2/5†	None	None
2	B-3	1302 A	+	1/5	4/5	None
3	B-3	1302 I	+	1/5	3/5	None
4	B-3	Dalldorf I	+	1/5	4/5	None
5	B-1	Conn 5 I	+	2/5	None	None
6	Saline	—	+	5/10	None	2/10‡
7	Saline	—	None	0/10	None	None

* Selected to include hearts *with* gross lesions in Groups 5 and 6 and *without* gross lesions in Group 2. Two longitudinal sections were made of each heart; lesions in either section scored heart as positive for necrosis.
† Number of hearts which showed lesions out of total number examined.
‡ In 2 hearts which showed necrosis.

survivors were killed and examined for gross cardiac lesions six days after inoculation. The protocol of this experiment is presented in Table VI. In this experiment a number of viral strains was employed in comparable dosage in an endeavor to answer the following questions: 1) Was the cardio-necrotic effect of 1302 virus limited to this strain or could necrosis be induced by another B-3 virus (Dalldorf strain)? 2) Might the infant mouse line of 1302 virus induce cardiac necrosis comparable to that associated with the adult mouse virus? 3) Could a Group B virus of another antigenic type (B-1, Conn. 5) induce myocardial necrosis? 4) Was "spontaneous" myocardial necrosis present in control mice which received neither cortisone nor virus?

In Table VI, the incidence and extent (score) of grossly discernible lesions in mice which received differing inocula are compared. It may be seen that: 1) Administration of virus (1302 adult) alone did not kill mice and induced gross necrosis in but one heart in this short observation period; 2) with cortisone and the same virus almost one-third of the animals had succumbed by the 6th day and three-fifths of survivors exhibited gross cardiac necrosis; 3) the infant mouse line of 1302 virus was rapidly lethal, and did, indeed, induce gross myocardial necrosis in almost half the survivors, but this necrosis was less than half as extensive as that associated with the adult mouse virus; 4) another Group B, type 3 virus was similarly cardio-necrotizing with cortisone; 5) Group B, type 1 virus (Conn. 5) was associated with necrosis of minimal extent in but three of 28 survivors; 6) only one animal which received cortisone without virus incurred a gross myocardial lesion; 7) no gross lesions were observed in mice which received neither virus nor cortisone.

The possibility was considered that grossly inapparent but microscopically detectable necrosis might be present in hearts of mice in the control groups which had received cortisone alone, or neither virus nor cortisone. Ten hearts from each of these groups were sectioned longitudinally at two levels and carefully studied for histological abnormalities. Results of this study are summarized in Table VII, together with the results of an examination of five hearts from each group which received a viral inoculum. It will be seen that disseminated myocarditis predominated in animals which received B-3 viruses and cortisone. The negligible cardio-necrotic effect of B-3 virus *without* cortisone or of B-1 virus even supplemented with cortisone was demonstrated, paralleling the gross observations. No necrosis was demonstrated in "normal" control mice. However, microscopy revealed that 50 per cent of hearts from mice which received only cortisone had small focal areas of necrosis, associated in two instances with the perivasculitis described earlier.

The extent of necrosis noted with B-1 virus and cortisone did not exceed that associated with cortisone alone so that present evidence suggests that B-1 virus, or at least the strain employed, does not possess the marked cardiopathogenicity which characterizes the three B-3 strains studied. Although some enhancement of the myocardial pathogenicity of the 1302 strain has attended its passage in the cortisone-injected adult mouse, it

is strongly suggested that the cardio-necrotic effect of the 1302 strain is related neither to its passage history nor to its characteristics as an isolate, but rather to the fact that it is a Group B, type 3 Coxsackie virus.

DISCUSSION

The present study has established beyond peradventure that gross myocardial necrosis may be effected by injection of cortisone into the adult mouse infected with a Coxsackie virus intrinsically capable of multiplication in the myocardium. Neither virus multiplication or cortisone administration alone induced lesions comparable in incidence or extent to those associated with administration of both virus and hormone. So too, the induction of lethal infection was dependent on the administration of cortisone. Although earlier studies have demonstrated the marked influence of cortisone on the multiplication of influenza B virus (11, 12), significant effects on multiplication of 1302 virus have not been shown. In contrast, studies with unadapted influenza B virus in mice have demonstrated the selection on passage of virus partially "dependent" on cortisone for both multiplication and pathogenicity (13). However, only the cardio-pathogenic effects of 1302 virus may be described as cortisone-dependent.

Delay of cortisone injection until the peak of influenza B virus increase in eggs results in diminished inflammation and prolonged survival (14), yet opposite effects are observed with 1302 virus infection and delayed cortisone treatment. This fact buttresses other evidence that necrosis is not the result of attainment of critical concentrations of cardiac virus *per se*, but is more directly effected by cortisone. In view of the occurrence of myocardial lesions associated with cortisone administration alone, it appears possible that cardiac necrosis results from the synergistic influence of virus and cortisone toxicity. Thus, histologically inapparent damage resulting from the insult of either agent alone may be compounded to overt necrosis in the presence of both in combination. This hypothesis is no less attractive if cortisone-induced focal necrosis is thought to result from activation of a latent infective agent *in situ*. Against this possibility are the apparent non-transmissibility of the disease in the present investigation and the occurrence of cortisone-induced damage in two species other than the mouse in skeletal muscle (15) as well as cardiac (7) sites. Skeletal muscle necrosis in the mouse has also been reported (16).

The apparent restriction of perivascular inflammation to the hearts of animals which received only cortisone is deserving of comment. It might be inferred that in some manner viral invasion "protects" the hearts of animals subjected also to cortisone administration. Such an inference might imply a viral etiology of cortisone-induced lesions and possible interference between viruses. This explanation would, of course, conflict with the hypothesis of synergistic damage advanced above, unless it were postulated that the qualitative nature of the pathologic reaction were modified by two agents in combination. A definitive answer to this question will depend on further study.

The demonstrated cardiotropicity of B-3 virus is of more than academic interest in view of the reports by Gear that a Coxsackie virus of the same antigenic type may induce fatal myocarditis in human infants (17, 18). It is not unreasonable to speculate on the possible conversion of the benign pleurodynia of adults to more serious, if not fatal, disease by the stimulus of endogenous or exogenous corticosteroids.

SUMMARY

1. The administration of cortisone to adult mice infected with Group B, type 3 Coxsackie virus results in extensive and disseminated myocardial necrosis.

2. The separate administration of virus or cortisone results in minimal and inconstant focal necrosis which is rarely evident on gross inspection.

3. In the absence of exogenous cortisone virus may multiply to a high concentration in the myocardium without inducing microscopically apparent pathologic change. Multiplication of the virus is not manifestly influenced by cortisone.

4. The gross necrosis associated with injection of virus and cortisone is prevented by concomitant injection of specific viral antiserum.

5. The induction of myocardial necrosis is not dependent on a virulent adaptation of virus to the adult mouse, but may be effected with virus previously passed only in infant mice.

6. Two different strains of Group B, type 3 virus were cardionecrotizing in contrast to the equivocal myocardial pathogenicity of a Group B, type 1 virus also capable of multiplication in the adult mouse.

7. It is postulated that the myocardial necrosis observed is not the indirect result of an influence of cortisone on viral multiplication, but rather the result of a synergistic toxicity of cortisone and an intrinsically cardiotropic virus.

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ASOLECTIN SEPARATION OF PROTHROMBIN FROM PROCONVERTIN

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All reported adsorbents of prothrombin simultaneously adsorb proconvertin, and practically all described techniques of elution have resulted in their simultaneous release from these adsorbents.

In this paper we present information about a material selectively removing prothrombin from an eluate containing prothrombin, proconvertin, and hemophilia B factor (PTC, Christmas factor). The substance exhibiting this unique behavior is asolectin.

In another communication (1) we have reported that suitable concentrations of asolectin used with the described technique selectively adsorb proaccelerin from citrated human plasma. Greater concentrations of asolectin, however, led to the loss of prothrombin and still greater concentrations to marked loss of fibrinogen reactivity. In both the latter instances, surprisingly, proconvertin activity was little reduced.

Accordingly, using the dialyzed eluate of washed barium sulfate that had adsorbed prothrombin, proconvertin, and hemophilia B factor from fresh oxalated normal human plasma, it was possible to demonstrate that selective, almost complete, removal of prothrombin activity by asolectin can be effected with only minor depletion of proconvertin activity. The ability of the asolectin treated eluate to shorten the partial thromboplastin time of hemophilia B plasma was moderately reduced.

MATERIALS

Oxalated normal platelet poor plasma was prepared as previously described (1). The plasma was harvested with a Pasteur pipette and used immediately for barium sulfate adsorption.

Barium sulfate (Baker) analytical grade.

0.9 per cent sodium chloride solution.

0.006 M sodium citrate solution.

0.14 M sodium citrate solution.

Asolectin (Associated Concentrates, Inc., 57–01 32nd Avenue, Woodside, New York) Lot Number 2085.

Parafilm.

Cellophane dialyzing membrane.

Russell viper venom (Stypven, Burroughs Wellcome and Company, London, England) diluted 1:40,000 in a 1:30 suspension of *cephalin* in veronal buffer was prepared by the technique of Hjort, Rapaport, and Owren (2) as was:

Prothrombin free ox plasma.

Citrated, proconvertin-deficient plasma was prepared from the blood of a patient congenitally deficient in this factor as previously described (3).

Citrated, hemophilia B plasma was similarly prepared from the blood of a patient with the classical laboratory and clinical findings of hemophilia B (PTC deficiency, Christmas disease). The supernatant plasma was transferred with a Pasteur pipette to a glass bottle, and glass-activated (4). It was then decanted and bottled in aliquots which were stored at minus 20°C. and thawed immediately before use.

Saline extract human brain thromboplastin was prepared as previously described (5).

Aged citrated human serum was prepared as described by Owren and Aas (6) and by Aas (7).

Calcium chloride aqueous solutions were prepared in the molarities indicated in the individual test systems.

Diluting fluid I and II were prepared as previously described (1).

Veronal buffer (pH 7.35, ionic strength 0.154) was prepared as described previously (2).

Buffer saline was prepared as previously described (1).

Adsorbed ox plasma was prepared as previously described (6) by passing fresh oxalated ox plasma once through a 20 per cent asbestos, then twice through a 50 per cent asbestos filter.

Accelerin-calcium chloride 30 mM was prepared by adding 0.5 ml. of thrombin (Hemoclaudan Adrenon Leo, Lövens Kemiske Fabrik, Köbenhavn, 100 units per ml. dissolved in veronal buffer) to 10 ml. of adsorbed ox plasma at 37°C. After 30 minutes' incubation, the fibrin was removed from the clotted plasma. Thrombin addition, incubation, and defibrination were repeated two more times. The resulting accelerin was then diluted with an equal volume of 60 mM calcium chloride solution to produce accelerin-calcium chloride 30 mM. Aliquots were stored at minus 20°C. and retained sufficient potency for use for periods of one week or more. During tests the accelerin-calcium chloride 30 mM was kept at 37°C.

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METHOD

Using a minor modification of the technique described by Surgenor, Alexander, Goldstein, and Schmid (8), 100 ml. of fresh oxalated normal plasma was mixed with 100 mg. of barium sulfate per ml. and mixed with a glass stirring rod for 30 minutes at 4°C. It was then centrifuged at 2500 rpm (2°C.) for 30 minutes. The barium sulfate sediment was drained and washed by mixing with 30 ml. physiological saline with a glass rod, then recentrifuged at 2500 rpm (2°C.) for 30 minutes. The saline supernate was decanted and discarded. The saline wash, centrifugation, and decanting were repeated. The barium sulfate was then stirred with 30 ml. of 0.006 M sodium citrate solution for 5 minutes and again centrifuged at 2500 rpm (2°C.) for 30 minutes. The wash fluid was decanted and the barium sulfate was stirred with 30 ml. of 0.14 M sodium citrate solution and allowed to elute for 30 minutes at 4°C. The inactive precipitate was sedimented by centrifugation at 2500 rpm (2°C.) for 30 minutes. The supernate was transferred to a cellophane membrane and dialyzed overnight against two 1-L. changes of physiological saline solution at 4°C. After saving a sample of the dialyzed eluate for initial assays, the remainder was transferred in aliquots to a mortar and 30 mg. of asolectin per ml. of eluate was ground with a pestle until it was evenly suspended. The suspension was placed in a glass tube and incubated at 4°C. for 60 minutes, then transferred to lusteroid tubes, capped, and spun at 40,000 rpm (2°C.) for 60 minutes. The tubes were removed from the rotor with utmost care to avoid agitating the contents. The central screw plug in the cap was removed, and a lumbar puncture needle attached to a 20-ml. Lur-lock syringe was carefully introduced to a point slightly above the tube bottom on the side opposite the sedimented asolectin. By gentle aspiration, the clear supernate was collected from each tube and pooled. A sample of this once-asolectin-treated eluate was saved for assay, and the balance was added in aliquots to a mortar containing 30 mg. of asolectin per ml. of once-asolectin-treated eluate. With a pestle, this was ground to an even suspension. The suspension was transferred to a glass tube and kept at 4°C. for 1 hour. It was then transferred to lusteroid tubes, capped, and spun at 40,000 rpm (2°C.) for 1 hour. Aspiration of the clear supernates was carried out and these pooled and labelled "twice-asolectin-treated eluate." All specimens were then subjected to assay.

All incubations and tests were performed in duplicate at 37°C.

The prothrombin assay systems of Hjort, Rapaport, and Owren (2) and of Owren and Aas (6) were both used.

The proconvertin assay system using plasma from a patient congenitally deficient in proconvertin as substrate was as previously described (1, 3).

Hemophilia B plasma partial thromboplastin time corrections:

Asolectin suspension (1:120 in buffer saline)	0.2 ml.
Citrated, glass-activated, Hemophilia B plasma, undiluted	0.2 ml.
Test material, undiluted	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Accelerin-calcium chloride 30 mM.	0.2 ml.

COMMENT

Prothrombin concentration is markedly reduced by treating an eluate rich in prothrombin and proconvertin with 30 mg. of asolectin per ml. Proconvertin activity is little reduced. A second treatment with 30 mg. of asolectin per ml. of the once-

TABLE I

Results *

Material tested	Proconvertin assay Duplicate determinations			Prothrombin assay [Hjort, Rapaport, and Owren (2)] Duplicate determinations		
	sec.	*sec.*	%	*sec.*	*sec.*	%
Initial eluate	14.4	14.8	>100	24.6	24.6	100
Once-asolectin-treated eluate	15.7	15.9	>100	76.4	77.2	19
Twice-asolectin-treated eluate	23.2	23.2	70	202.4	204.0	0.5

* Twice-asolectin-treated eluate (diluted 1 : 10 in diluting fluid II) when tested in the aged citrated human serum method for prothrombin assay (6) gave the following times: 83.7, 83.0 (<1 per cent) as contrasted with 98.2, 93.5 for buffer (0 per cent) control.

In the hemophilia B partial thromboplastin time correction using 0.2 ml. hemophilia B plasma as substrate, 0.2 ml. of the following undiluted materials added as test substances gave the recorded results from the time accelerin-$CaCl_2$ 30 mM was added:

	Duplicate determinations	
	sec.	*sec.*
0.2 Hemophilia B plasma	264.7	266.2
0.2 Initial eluate	25.4	27.4
0.2 Once-asolectin-treated eluate	45.9	44.6
0.2 Twice-asolectin-treated eluate	56.3	56.1
0.2 Normal plasma	45.2	46.7

treated eluate leaves only traces of prothrombin behind, while proconvertin activity is reduced to only 70 per cent of normal plasma's content. Hemophilia B factor activity seems to be reduced only moderately. It would appear that the sodium citrate eluate of barium sulfate adsorbent of fresh oxalated hemophilia B plasma or asolectin treated hemophilia B serum should necessarily be used if one wishes to prepare proconvertin free of prothrombin *and* hemophilia B factor.

Attempts to elute prothrombin from the asolectin with 0.14 M sodium citrate, with 0.9 per cent sodium chloride, with distilled water, with 1 per cent acetic acid, and with phosphate buffer (pH 8.0) have not succeeded. Attempted solution of asolectin and precipitation of prothrombin by adding a 10 per cent volume of ethyl ether (4°C.), attempts to achieve differential solubility of prothrombin and asolectin in tri-fluoro acetic acid, and attempts to oxalate and competitively adsorb prothrombin on barium sulfate from the asolectin and elute it from the barium sulfate have all been unsuccessful. Efforts to separate the prothrombin are continuing. The probability that the bond between asolectin and prothrombin may be chemical rather than physical is recognized.

SUMMARY

Asolectin 30 mg. per ml. ground to an even suspension in a mortar and pestle with a dialyzed sodium citrate eluate of the barium sulfate adsorbent of fresh oxalated normal human plasma is capable of selectively and almost completely removing prothrombin activity. Proconvertin activity and the ability of the treated eluate to shorten the partial thromboplastin time of hemophilia B plasma are reduced to a much lesser degree. The asolectin suspension is incubated with the eluate for 60 minutes at 4°C., then sedimented by centrifugation at 40,000 rpm for 60 minutes (2°C.).

Attempts to elute the prothrombin from asolectin have been unsuccessful to date.

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EFFECT OF A PHENYLBUTAZONE ANALOG (4-[PHENYLTHIOETHYL]-1,2-DIPHENYL 3,5-PYRAZOLIDINEDIONE) ON URATE CLEARANCE AND OTHER DISCRETE RENAL FUNCTIONS IN GOUTY SUBJECTS. EVALUATION AS URICOSURIC AGENT[1]

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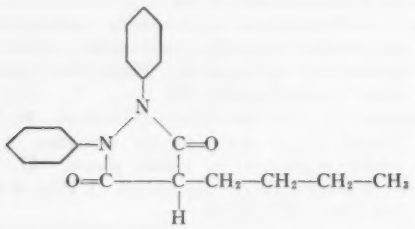
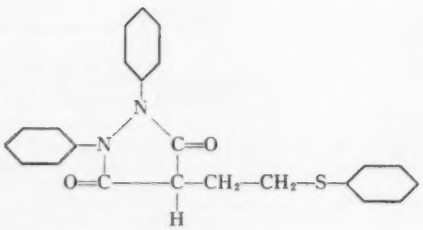
A number of phenylbutazone derivatives and metabolites are under study (1–4) in a joint effort to develop an analog possessing the marked anti-rheumatic properties but not the undesirable side effects of the parent compound. In the course of testing the anti-rheumatic effects in acute gouty arthritis of one of these compounds, G–25671, in which a phenylthioethyl group replaces the butyl side chain of phenylbutazone, marked uricosuric activity was noted (1, 2). The interesting properties of this substance suggested further study and a clinical trial in the management of gout.



Phenylbutazone



G-25671

4-(phenylthioethyl)-1,2-diphenyl 3,5-pyrazolidinedione

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The present report deals with the effect of G–25671 on the simultaneous renal clearances of inulin, para-aminohippurate, urate, sodium, potassium and chloride. More protracted urate and electrolyte balance studies are included. Our clinical experience with G-25671 in gouty subjects also is briefly summarized.

METHODS

Renal clearance studies were carried out in 12 gouty subjects, all males 33 to 62 years of age, and in the intercritical phase of the disorder. These subjects had been maintained on a low purine, low fat, restricted protein (60 to 75 gm. per day) diet and were in the post-absorptive state at the time of study. Standard clearance techniques (5) were employed, with slight modifications described elsewhere (6).

To ensure adequate urine flow throughout the experiments, liberal quantities of water were administered orally. Urine and blood collections were initiated in three pre-medication control periods, each of 20 to 30 minutes duration. Then G–25671 was injected intravenously, the rate of injection being varied according to the design of the experiment, and appropriate urine and blood collections were made.

In 7 subjects the total drug dosage, 0.90 to 1.54 gm. of the sodium salt (16 mg. per Kg. body weight) dissolved in 100 to 150 ml. isotonic saline solution, was delivered within a period of approximately 20 minutes to attain high blood levels of G–25671 rapidly. Injection of the drug was then discontinued and 4 to 6 urine collections were obtained, each comprising a period of 20 to 30 minutes. Three blood samples were procured during these collection periods. The respective plasma concentrations of inulin, PAH, urate, Na, K and Cl were obtained by plotting the concentrations semilogarithmically against time and by extrapolating back to 2.5 minutes (approximate urine delay time) from the mid-time of each urine collection period. In three of these subjects, A. R., M. C., and L. W., parallel clearance studies with phenylbutazone,

as the sodium salt, were made. In Cases A. R. and L. W. the effects of injection of 100 ml. saline solution alone also were determined.

Five experiments were designed to study the effects of slowly rising drug levels to determine the order of effects on specific renal clearances. In Cases J. S. and L. L., G-25671 as the sodium salt was dissolved in 5 per cent glucose in water to make a 0.14 per cent solution of the drug which was then delivered by sustained intravenous injection. The initial rate of infusion was 1 ml. per minute; this was increased by an increment of approximately 1 ml. per minute at the beginning of each 20-minute urine collection period to make a total drug dosage of 0.80 gm. for Case J. S. and 0.86 gm. for Case L. L. Seven such collections were made in each experiment. Blood samples were procured at the mid-point of each clearance period. Parallel experiments were carried out in Cases N. P., J. B., and L. O., in which phenylbutazone as the sodium salt dissolved in 5 per cent glucose in water to make a 0.14 per cent solution of the drug, was delivered by slow infusion.

To obtain information as to the more protracted effects of G–25671, particularly on the renal excretion of sodium, potassium, chloride and inorganic phosphate, two gouty subjects were hospitalized for two weeks during which they were maintained on constant weighed diets

TABLE I

*Effect of rapid infusion of G-25671 (in isotonic saline solution) on renal clearances of inulin, PAH and urate—Three analogous experiments with phenylbutazone are included**

Case Age B.S.A.	Period	$P_{G-25671}$ or P_{Pb}	C_{In}	C_{PAH}	P_{Urate}	C_{Urate}	$\frac{C_{Urate}}{C_{In}}$	Remarks
		mg. %	*ml./min.*	*ml./min.*	*mg. %*	*ml./min.*	%	
1. A. R.	Control	0	132	511	8.7	7.84	5.93	Non-tophaceous gout, 11 years; no clinical evidence of cardiorenal disease.
44	G-25671: 1.4 gm. in 150 ml. isotonic saline solution							
(2.05 M²)	0–39 min.	19.0	106	*355*	8.4	17.6	16.6	
	39–67 min.		119	407	7.8	47.0	39.5	
	67–92 min.	13.4	115	397	7.5	45.5	*39.6*	
	92–122 min.		134	421	7.0	*50.7*	37.8	
	122–152 min.	11.2	104	356	6.4	41.2	39.6	
	Control	0	117	551	7.9	6.35	5.30	
	Phenylbutazone: 1.4 gm. in 150 ml. isotonic saline solution							
	0–34 min.	11.2	122	445	7.8	13.1	10.8	
	34–62 min.		103	417	7.8	*22.5*	*21.8*	
	62–90 min.		102	*398*	7.7	14.0	13.7	
	90–113 min.	9.9	129	465	7.6	15.8	12.3	
	113–127 min.	9.7	137	515	7.4	15.6	11.4	
2. L. W.	Control	0	104	529	8.6	3.38	3.25	Non-tophaceous gout, 12 years; no clinical evidence of cardiorenal disease.
46	G-25671: 1.2 gm. in 100 ml. isotonic saline solution							
(1.93 M²)	0–32 min.	22.7	72.0	*287*	8.2	10.1	14.0	
	32–50 min.		81.2	375	7.5	26.3	32.4	
	50–73 min.		119	493	7.0	47.8	40.1	
	73–92 min.		103	507	6.6	*50.8*	*49.3*	
	90–107 min.	15.7	97.0	500	6.3	47.5	49.0	
	Control	0	109	404	7.0	3.92	3.60	
	Phenylbutazone: 1.2 gm. in 100 ml. isotonic saline solution							
	0–33 min.	13.8	119	*293*	6.7	10.3	8.70	
	33–61 min.		112	356	6.6	*19.9*	*17.8*	
	61–88 min.		107	325	6.6	12.1	11.3	
	88–109 min.	12.0	110	332	6.6	11.2	10.2	
	109–133 min.	11.5	101	294	6.4	11.4	11.3	
3. M. C.	Control		124	590	8.6	7.62	6.14	Non-tophaceous gout, 1 year; no clinical evidence of cardiorenal disease.
33	G-25671: 1.3 gm. in 100 ml. isotonic saline solution							
(1.93 M²)	0–25 min.		118	*451*	8.5	18.3	15.5	
	25–48 min.		125	462	8.3	*36.5*	29.2	
	48–71 min.		115	517	8.2	36.2	*31.5*	
	71–100 min.		122	531	8.1	35.6	29.2	
	100–122 min.		117	474	7.9	32.9	28.1	
	122–143 min.		130	493	7.7	35.2	27.1	
	Control	0	119	600	9.8	6.55	5.00	
	Phenylbutazone: 1.3 gm. in 100 ml. isotonic saline solution							
	0–35 min.	13.6	114	*440*	9.8	9.28	8.10	
	35–77 min.		145	538	9.3	8.62	6.00	
	77–107 min.		141	608	9.0	10.2	7.20	
	107–130 min.	11.5	149	564	9.0	*13.4*	*9.00*	
	130–151 min.	11.0	122	450	9.1	9.42	7.70	

TABLE I—*Continued*

Case Age B.S.A.	Period	$P_{G-25671}$	C_{In}	C_{PAH}	P_{Urate}	C_{Urate}	$\frac{C_{Urate}}{C_{In}}$	Remarks
		mg. %	*ml./min.*	*ml./min.*	*mg. %*	*ml./min.*	*%*	
4. M. H.	Control	0	92.4	416	9.9	4.88	5.28	Tophaceous gout, 17 years; no clinical evidence of cardiorenal disease.
45	G-25671: 0.95 gm. in 100 ml. isotonic saline solution							
(1.68 M²)	0–34 min.	12.3	93.4	334	9.6	11.5	12.3	
	34–54 min.		96.0	337	9.3	28.8	30.0	
	54–79 min.	10.7	99.0	343	9.0	29.6	29.9	
	79–104 min.		104	337	8.6	*32.9*	*31.7*	
	104–130 min.	8.4	96.3	275	8.4	22.1	23.0	
	130–152 min.		92.0	*223*	8.3	25.2	27.4	
5. S. S.	Control	0	86.0	453	9.7	6.14	7.13	Non-tophaceous atypical gout; no clinical evidence of cardiorenal disease.
38	G-25671: 1.0 gm. in 100 ml. isotonic saline solution							
(1.66 M²)	0–35 min.	16.7	90.2	424	9.0	4.90	5.43	
	35–59 min.		88.4	*413*	8.4	8.60	9.73	
	59–90 min.		78.9	429	8.2	14.3	18.1	
	90–120 min.	12.0	82.0	473	7.9	*17.8*	*21.7*	
6. H. G.	Control	0	83.7	290	11.5	6.68	7.97	Tophaceous gout, 11 years; post-scarlatina nephritis for more than 20 years; albuminuria and cylindruria, serum NPN 34 mg. %, PSP excretion 50% in 2 hours.
44	G-25671: 1.5 gm. in 150 ml. isotonic saline solution							
(2.08 M²)	0–43 min.	19.1	83.6	*165*	11.1	*13.8*	*16.5*	
	43–75 min.		75.2	183	10.8	9.40	12.5	
	75–100 min.		74.8	183	10.5	10.3	13.8	
	100–123 min.		72.9	362	10.2	10.8	14.8	
	123–153 min.	11.1	68.0	333	10.2	11.0	16.2	
7. I. K.	Control		64.9	202	8.7	4.76	7.34	Non-tophaceous gout 32 years; nephrosclerosis; albuminuria and cylindruria, serum NPN 60 mg. %, PSP excretion 50% in 2 hours; post-gastrectomy refractory anemia, 15 years.
62	G-25671: 0.9 gm. in 100 ml. isotonic saline solution							
(1.65 M²)	0–37 min.		64.3	*147*	8.4	9.63	15.0	
	37–62 min.		66.7	156	8.2	11.5	17.3	
	62–88 min.		80.0	198	8.2	15.5	19.3	
	88–115 min.		79.4	208	8.2	*18.7*	*23.6*	

* Figures italicized indicate maximal changes.

and controlled salt and water intake. The daily caloric intake for subject O. G. was 1600 (protein, 60 gm. per day), with 2.5 gm. of sodium chloride added; subject L. W. received 1800 calories per day (protein, 60 gm. per day) with 3.5 gm. sodium chloride added. On the seventh day of hospitalization, G-25671 administration was begun: O. G. received 1.2 gm. intravenously every day for four consecutive days, delivered in three divided doses of 0.4 gm. (in 100 ml. 5 per cent glucose in distilled water) at 9:00 A.M., 3:00 P.M., and 9:00 P.M.; L. W. received 1.6 gm. intravenously for four consecutive days, similarly delivered daily in three divided doses of 0.4 gm. (9:00 A.M.), 0.4 gm. (3:00 P.M.) and 0.8 gm. (9:00 P.M.). Blood samples were secured just prior to each infusion. Urine collections, obtained by spontaneous voidings, were spaced to comprise the periods from 9:00 A.M. to 3:00 P.M., then to 9:00 P.M., and overnight to 9:00 A.M. The urine was collected over the three days immediately preceding medication, the four days of G–25671 administration, and three days thereafter. The body weight was recorded each morning. Parallel studies with phenylbutazone had previously been made in these two subjects (7).

The analytical methods for the determination of urate, inulin, creatinine, para-aminohippurate, sodium, potassium, chloride, and inorganic phosphate were the same as previously employed (6). Plasma concentrations of G–25671 were measured by the method previously described (8) for phenylbutazone except that 3 per cent isoamyl alcohol was added to the petroleum ether used for extraction of the drug and the optical densities were measured at 255 mμ.

RESULTS

Plasma levels of G–25671 after intravenous injection

The plasma levels of G–25671 obtained when the total dosage of drug (16 mg. per Kg. body weight) was delivered in one rapid injection varied from 22.7 to 12.3 mg. per cent at the mid-point of the first post-medication collection period (Table I). In every instance the plasma concentration of the drug declined in the course of the experiment, the final concentrations ranging from 15.7 to 8.4 mg. per cent. This is in contrast to the very slow disappearance of phenylbutazone from the plasma (7).

In two experiments involving sustained infusion of the drug at increasing rates (Table II), the plasma concentration of G–25671 was slowly

increased from initial trace levels to 9.5 and 5.4 mg. per cent, respectively, in Cases J. S. and L. L.

Effects of G–25671 on C_{In} and C_{PAH}

In Cases A. R., L. W., and M. C., the values for C_{In} and C_{PAH} prior to injection of G–25671 were within the normal range (Table I). The remaining six subjects were found to have significant reductions in glomerular filtration rate and/or renal plasma flow (Tables I and II), as commonly observed in older patients with a long history of overt gout. Cases J. S. and I. K. exhibited the most marked impairment of renal hemodynamics (C_{In} 54.4 and 64.9 ml. per min., C_{PAH} 242 and 202 ml. per min., respectively).

Infusion of G–25671 in the dosage employed did not significantly affect the glomerular filtration rate in five subjects (Tables I and II). In three instances C_{In} was appreciably although transitorily depressed, to a maximum of − 30 per cent in

TABLE II

Effect of slow, sustained infusion of G-25671 and of phenylbutazone (in glucose solution) on renal clearances of inulin, urate, sodium, potassium and chloride

Case Age B.S.A.	Period	$P_{G-25671}$ or P_{Ph}	C_{In}	P_{Urate}	C_{Urate}	$\frac{C_{Urate}}{C_{In}}$	UV_{Na}	C_{Na}	UV_K	C_K	UV_{Cl}	C_{Cl}
		mg. %	*ml./ min.*	*mg. %*	*ml./ min.*	*%*	*mEq./ min.*	*ml./ min.*	*mEq./ min.*	*ml./ min.*	*mEq./ min.*	*ml./ min.*
1. J. S.	Control	0	54.4	11.0	4.81	8.91	0.42	3.01	0.062	18.2	0.21	2.00
60	G-25671: 0.80 gm. (9.5 mg./Kg. body weight) in 5% glucose/water											
(1.98 M²)	0–25 min.	Trace	47.0	11.0	3.51	7.47	0.39	2.76	0.060	14.6	0.18	1.64
	25–45 min.	0.6	49.2	11.0	3.84	7.80	0.40	2.84	0.050	12.6	0.18	1.69
	45–68 min.	1.3	60.5	11.0	8.22	13.6	0.39	2.77	0.049	12.5	0.21	2.01
	68–90 min.	4.0	60.5	11.0	8.86	14.7	0.38	2.70	0.043	11.0	0.19	1.81
	90–109 min.	5.2	63.9	10.7	11.8	18.5	0.33	2.37	0.039	10.0	0.18	1.68
	109–128 min.	7.3	60.3	10.3	16.0	26.6	0.33	2.34	0.033	9.4	0.15	1.40
	128–145 min.	9.5	56.5	10.3	16.8	29.7	0.29	2.10	0.029	8.6	0.12	1.13
2. L. L.	Control	0	89.1	7.5	9.54	10.7	0.28	2.07	0.082	20.6	0.27	2.62
60	G-25671: 0.86 gm. (11.5 mg./Kg. body weight) in 5% glucose/water											
(1.87 M²)	0–24 min.	Trace	82.3	8.0	9.60	11.7	0.24	1.78	0.064	15.9	0.24	2.34
	24–45 min.	0.2	82.3	7.8	9.28	11.3	0.28	2.06	0.079	19.7	0.28	2.72
	45–66 min.	1.0	92.8	7.6	11.4	12.3	0.29	2.12	0.081	20.2	0.29	2.82
	66–89 min.	1.2	90.0	7.3	12.6	14.0	0.30	2.16	0.073	18.2	0.28	2.78
	89–109 min.	2.7	92.8	7.4	16.3	17.6	0.30	2.18	0.067	16.6	0.28	2.72
	109–130 min.	3.7	91.1	8.1	19.5	21.4	0.31	2.27	0.064	16.0	0.27	2.62
	130–153 min.	5.4	92.5	8.0	23.0	24.8	0.31	2.25	0.058	14.5	0.26	2.52
3. N. P.	Control	0	81.3	10.6	1.52	1.87	0.10	0.71	0.021	5.53	0.072	0.74
43	Phenylbutazone: 0.46 gm. (5.1 mg./Kg. body weight) in 5% glucose/water											
(2.13 M²)	0–36 min.	Trace	88.0	10.5	1.24	1.41	0.10	0.70	0.023	6.05	0.073	0.75
	36–57 min.	0.5	75.0	10.1	1.07	1.43	0.082	0.57	0.022	5.80	0.060	0.62
	57–80 min.	0.8	88.9	10.5	1.12	1.26	0.082	0.57	0.024	6.32	0.057	0.59
	80–99 min.	2.3	99.3	10.5	1.38	1.39	0.084	0.59	0.027	7.11	0.056	0.57
	99–120 min.	4.0	89.4	10.5	1.09	1.22	0.069	0.48	0.026	6.86	0.043	0.44
	120–135 min.	3.5*	89.4	10.5	1.26	1.41	0.081	0.56	0.026	6.86	0.050	0.51
4. J. B.	Control	0	127	8.5	6.67	5.22	0.21	1.45	—	—	0.17	1.62
56	Phenylbutazone: 0.56 gm. (8.2 mg./Kg. body weight) in 5% glucose/water											
(1.74 M²)	0–26 min.	Trace	119	8.4	5.83	4.89	0.19	1.30	—	—	0.16	1.53
	26–51 min.	0.4	130	8.4	6.40	4.92	0.19	1.30	—	—	0.18	1.70
	51–85 min.	0.8	127	8.4	6.39	5.03	0.16	1.10	—	—	0.13	1.22
	85–106 min.	1.3	120	8.2	6.66	4.72	0.12	0.86	—	—	0.077	0.73
	106–128 min.	—	109	8.2	6.25	5.73	0.12	0.85	—	—	0.076	0.72
	128–176 min.	4.9	118	8.0	6.85	5.80	0.12	0.84	—	—	0.059	0.56
5. L. O.	Control	0	129	9.4	7.40	5.73	0.26	1.81	0.093	22.1	0.28	2.78
37	Phenylbutazone: 0.82 gm. (11.0 mg./Kg. body weight) in 5% glucose/water											
(1.90 M²)	0–25 min.	0.3	108	9.3	5.99	4.47	0.22	1.52	0.085	20.2	0.27	2.67
	25–47 min.	1.2	116	9.1	5.30	4.17	0.17	1.21	0.083	19.8	0.19	2.00
	47–68 min.	2.1	116	9.2	4.43	3.48	0.12	0.83	0.065	15.5	0.13	1.36
	68–90 min.	3.7	116	9.1	4.98	3.92	0.12	0.83	0.064	15.2	0.11	1.21
	90–122 min.	5.5	106	9.3	4.58	3.94	0.10	0.72	0.045	10.7	0.086	0.93
	122–154 min.	8.7	115	9.4	6.70	5.32	0.11	0.76	0.042	10.0	0.087	0.94

* Phenylbutazone stopped after 120 minutes.

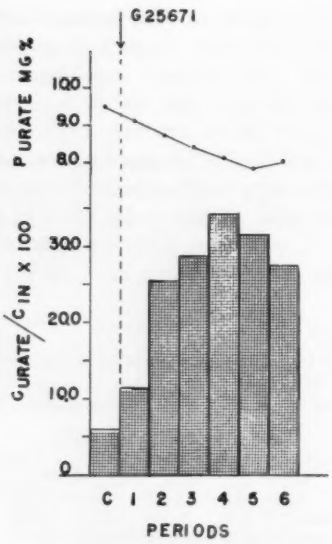

FIG. 1. EFFECT OF RAPID INTRAVENOUS INFUSION OF G–25671, IN 16 MG. PER KG. BODY WEIGHT DOSAGE, ON URATE CLEARANCE AND SERUM URATE LEVELS

The bars represent the means of seven urate/inulin clearance ratio determinations before injection (C) and in six post-injection collection periods. The line above represents the concomitant mean serum urate response in these seven experiments.

Case L. W., − 20 per cent in Cases A. R. and H. G. A pyrogenic reaction to the infusion in Case I. K. was associated with a transient rise in C_{In}. The overall change in C_{In} following G–25671 infusion was negligible in most instances. Phenylbutazone in comparable dosage likewise usually has little or no effect on the glomerular filtration rate but may depress C_{In} in some instances, more consistently when larger doses are employed (7).

C_{PAH} was rapidly and consistently depressed by G–25671 (Table I), the mean control value of 402 ml. per min. falling to a mean minimum of 284 ml. per min., an average decline approximating 30 per cent. This effect is presumed to be due to decreased renal extraction of PAH rather than to reduction in renal blood flow, since phenylbutazone (7) markedly depresses Tm_{PAH}. Both drugs significantly suppress the rate of phenolsulfonphthalein excretion (1), another indication of depression of tubular excretory mechanisms.

Effect of G–25671 on urate clearance

A marked increase in urate clearance occurred in every instance upon exhibition of the drug and was invariably apparent in the first collection period (Table I). The mean C_{urate}/C_{In} ratio rose from a pre-medication value of 6.15 per cent to a five-fold peak of 30.6 per cent. This effect was more pronounced in the subjects with normal renal hemodynamics (Cases A. R., L. W., and M. C.) whose C_{urate}/C_{In} ratio rose from a mean of 5.11 per cent to a mean peak of 40.1 per cent but was readily demonstrable also in those with depressed C_{In} and C_{PAH}, whose mean pre-medication C_{urate}/C_{In} ratio of 6.93 per cent increased to 23.4 per cent (Table I). The uricosuria induced by injection of G–25671 was associated invariably and roughly proportionately with a progressive decline in serum urate concentration, from a mean initial level of 9.3 mg. per cent to a mean minimum of 8.0 mg. per cent at the end of the period of observation (Figure 1). The fall in serum urate in individual patients ranged from 2.3 to 0.5 mg. per cent.

The data in Table II indicate that G–25671 begins to exert its uricosuric effect at low plasma concentrations of the drug, approximating 1 mg. per cent, and that this action is progressively enhanced as the drug levels rise.

The uricosuric action of G–25671 is attributable to suppression of tubular reabsorption of urate. The percentage of filtered urate which was reabsorbed (*i.e.*, filtered − UV/filtered) was calculated to fall, under the influence of the drug, from a mean pre-medication value of 93.9 per cent to a mean minimum of 70.2 per cent (in Case L. W., to as low as 50.8 per cent). As already implied, the quantity of urate filtered at the glomerulus was not significantly altered by the drug; no significant change in the proportion of plasma urate which is filtrable need be assumed (9); and the rapidity of the uricosuric action precluded any appreciable urate moiety due to augmented metabolic degradation of nucleic acids.

Effect of G–25671 on clearance and excretion (UV) of sodium, potassium and chloride

The experiments summarized in Table I included simultaneous determinations of C_{Na}, C_K and C_{Cl}. The results (data not included in Table

I) indicated an increase in sodium clearance in four of the seven subjects studied, no significant change in two, and a decrease in one; these alterations did not correlate with any changes in glomerular filtration rate. C_{Cl} paralleled C_{Na} throughout. C_K/C_{In} ratios were not significantly altered.

The increased sodium and chloride excretion noted in four subjects does not, under the conditions of these experiments, demonstrate that G–25671 may elicit sodium chloride diuresis since 14.2 to 24.0 mEq. of sodium, as sodium chloride solution and sodium salt of G–25671, were infused concomitantly with the drug. Calculation of the net electrolyte balance in fact revealed some retention of sodium, from 12.1 to 26.7 mEq. over the approximately 2-hour period of the experiment, in every patient except I. K. who developed a pyrogenic reaction. In Cases A. R. and L. W. (who received equivalent infusions of sodium chloride with and without the drug) 26.7 and 6.0 mEq. sodium, respectively, were retained when the drug, in saline solution, was infused, and 14.7 and − 1.6 mEq. sodium when sodium chloride alone was injected.

In two clearance experiments summarized in Table II, G–25671 was infused in a 5 per cent solution of glucose in water. Case J. S. shows retention of sodium chloride, Case L. L. does not. In both instances UV_K and C_K fell significantly; this result, which was not obtained in any experiments involving saline solution as a vehicle for G–25671, is attributed to the infusion of glucose and the utilization of potassium for glycogen formation.

Balance studies: G–25671 given by repeated intravenous injection

The observations made by the use of clearance techniques were supplemented by more protracted urate and electrolyte balance studies. In two such experiments G–25671 was administered intravenously in daily doses of 1.2 gm. (Case O.G.) and 1.6 gm. (Case L. W.), given in three divided doses, over a four-day period (Figure 2). These doses brought the plasma drug level in Case O. G. to 6.0 mg. per cent after 24 hrs. and to 11.8 mg. per cent on the fourth day of medication. In Case L. W. the plasma drug levels were 4.0 mg. per cent and 9.7 mg. per cent, respectively.

Patient O. G., whose mean urinary urate excretion was 408 mg. per 24 hrs. in the 3-day control period, increased his mean daily output to 626 mg. per 24 hrs. during the period of drug injection, a total excess urinary urate excretion of 872 mg. for the 4-day period of drug administration. This was associated with a sharp fall in serum urate, from 9.7 mg. per cent to 2.6 mg. per cent. Upon discontinuance of medication the urinary urate excretion fell below pre-medication levels to a mean of 270 mg. per 24 hrs.; this period of urate retention was associated with a slow rise in serum urate level to 6.7 mg. per cent after 3 days. Patient L. W. excreted a mean of 454 mg. urate per 24 hrs. in the control period. On the first day of drug injection the urinary urate output rose to

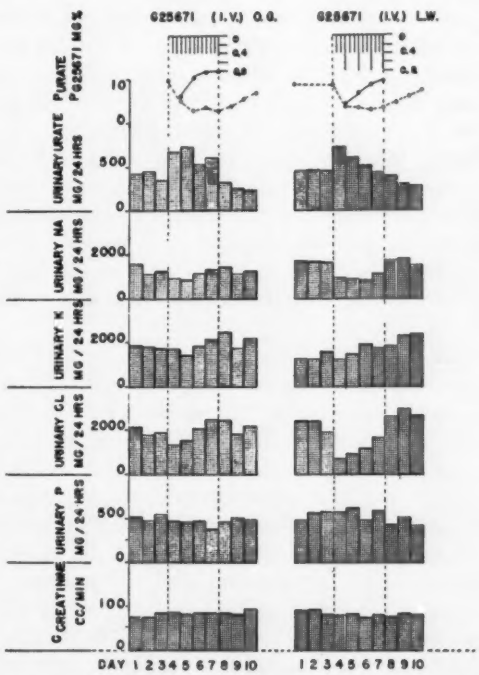
G25671 (I.V.) O.G.
G25671 (I.V.) L.W.
PURATE P G25671 MG%
URINARY URATE MG/24 HRS
URINARY NA MG/24 HRS
URINARY K MG/24 HRS
URINARY CL MG/24 HRS
URINARY P MG/24 HRS
G CREATININE CC/MIN
DAY 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

FIG. 2. CASES O. G. AND L. W. URINARY EXCRETION PER 24 HRS. OF URATE, SODIUM, POTASSIUM, CHLORIDE AND INORGANIC PHOSPHATE BEFORE, DURING, AND AFTER DAILY INTRAVENOUS INJECTION OF G–25671 IN THE DOSAGE INDICATED AT THE TOP

The effect on "endogenous" creatinine clearance is indicated at the bottom of the figure, the serum urate levels (hollow dots) and serum G–25671 (solid dots) above.

TABLE III

Effect of G-25671 on serum and urinary urate in five gouty subjects (Dosage 1.0 gm. per day)

Case	Uric acid	Pre-medication	Day of medication							Day of post-medication	
			1	2	3	4	5	6	7	1	2
1. J. Z.	Serum (mg./100 ml.)	9.1	6.6	4.3	3.0	3.0		3.7	3.4	5.0	5.7
	Urine (mg./24 hrs.)	604	1,250	1,337	1,095	818	717	785	805	560	393
2. R. C.	Serum	11.3	10.0	7.8	7.9	7.8	7.1		7.3	9.8	12.0
	Urine	852	975	1,597	1,476	1,133	1,158	1,409	1,450	848	654
3. A. K.	Serum	12.6	10.6	5.8	5.4		5.6			7.6	11.4
	Urine	493	911	1,268	910		922			576	512
4. R. E.	Serum	11.1	9.6	6.5	7.5	5.4				6.2	7.1
	Urine	424	606	866	702	531				316	487
5. L. C.	Serum	9.2	7.4	5.6	4.8					5.8	7.1
	Urine	671	972	938	938					605	487

736 mg. per 24 hrs., then declined to 596 mg. per 24 hrs. and thereafter to control levels—a temporary uricosuria often encountered when the quantity of mobilizable urate deposit is small and readily depleted, as in this instance. The serum urate level, initially 8.7 mg. per cent, fell to approximately 3.0 mg. per cent and remained there throughout the period of drug administration. This is an indication of continued suppression of tubular reabsorption of urate, despite the return of urinary urate excretion levels to pre-medication values. Discontinuance of the drug was followed by urinary urate retention and slow rise of the serum urate level.

In Case O. G. the mean control level of urinary sodium excretion, 1.32 gm. per 24 hrs., was reduced to a mean of 0.95 gm. per 24 hrs. for the first two days of drug administration, then reverted to the control figures; the urinary chloride excretion closely paralleled these changes. Case L. W., whose sodium chloride intake and drug dosage were greater than in Case O. G., showed more marked and sustained sodium chloride retention. The mean urinary sodium excretion in the control period, 1.69 gm. per 24 hrs., fell to a mean of 0.96 gm. per 24 hrs. during the 4-day period of drug administration. The urinary chloride excretion fell concomitantly to a mean of approximately 50 per cent of the mean control period levels. The urinary excretion of potassium, inorganic phosphate and creatinine was not significantly altered by G–25671.

The serum levels of sodium, potassium, chloride and inorganic phosphate remained essentially unchanged throughout the experiments. There was no significant decrease in the hematocrit in either patient although Case L. W. gained two pounds in weight.

Effect of oral administration of G–25671 on urinary urate excretion and serum urate levels

Table III records the day-by-day uricosuric response to daily doses of 1.0 gm. G–25671, given in five divided doses of 0.2 gm., in 5 gouty subjects maintained on a constant diet low in purines and fats, and containing 60 to 80 gm. protein per day. The mean increase in urinary urate excretion with this dosage was 55 per cent at the end of the first 24-hours of medication, reached a peak of 97 per cent after 48 hours, and continued to be augmented so long as the drug was administered. There was a concomitant sharp and sustained fall in serum urate. Cessation of medication was invariably followed by a period of urate retention.

Data on the response of the serum urate level are available in 13 gouty subjects, free of significant impairment of renal function, after oral administration of G–25671 in 1.0 gm. daily dosage. The mean pre-medication figure of 9.6 mg. per cent was reduced to a mean minimum of 4.7 mg. per cent.

A special study was made of the uricosuric response to G–25671 of gouty subjects with marked renal damage, since it is this category

which is most apt to be refractory to uricosuric agents, even in doses larger than ordinarily employed. Observations were made in five such patients after 1.0-gm. daily dosage and, in all but one, subsequently after 2.0-gm. daily dosage. In Case J. D., the initial urinary urate excretion of 656 mg. per 24 hrs. and serum urate level of 10.8 mg. per cent changed, with 1.2 gm. G–25671 daily, to 834 mg. per 24 hrs. and 7.7 mg. per cent, respectively, and then to 1140 mg. per 24 hrs. and 5.9 mg. per cent with 2.0-gm. daily dosage. In Case S. R., the respective figures were 968 mg. per 24 hrs. and 14.7 mg. per cent before medication; 1332 mg. per 24 hrs. and 7.2 mg. per cent with 1.2 gm. drug daily; 1145 mg. per 24 hrs. and 5.2 mg. per cent with 2.0 gm. drug daily. In Case S. T., the initial serum urate level of 10.6 mg. per cent fell to 9.7 mg. per cent on 1.2-gm. daily dosage and to 7.6 mg. per cent on 2.0-gm. daily dosage. In Case L. M., the response was altogether unsatisfactory: With 1.2 gm. G–25671 daily both urinary urate excretion and serum urate level were essentially unchanged from the initial figures of 805 mg. per 24 hrs., and 12.6 mg. per cent, respectively, and an attempt to increase the dosage had to be discontinued because of the appearance of a drug rash. Case G. S., who gave a typical history of chronic glomerulonephritis (with serum NPN 91 mg. per cent, PSP excretion 20 per cent in 2 hours) as well as gout, also responded unsatisfactorily. The pre-medication urinary urate excretion of 288 mg. per 24 hrs. was unchanged with 1.2-gm. daily dosage although the serum urate level fell from 11.1 to 9.4 mg. per cent; on 2.0-gm. daily dosage the urinary urate excretion was 228 mg. per 24 hrs. and the serum urate 7.7 mg. per cent.

Anti-rheumatic action

Animal screening tests indicate that G–25671 possesses marked anti-inflammatory properties in experimental inflammations.[2] This action is demonstrable also in man but the effects are distinctly less marked and less consistent than with phenylbutazone (1). Thus of 10 acute gouty attacks treated with 1.0 gm. G–25671 daily, only three remitted completely within 24 to 48 hours, two within 72 hours, four improved temporarily but recurred promptly upon discontinuance of the drug, and in one instance the dosage had to be increased to 2.0 gm. per day because of inadequate response. All these patients showed the usual prompt and marked uricosuria, with decline in serum urate levels.

These results discouraged further trial of G–25671 in the termination of severe attacks of acute gouty arthritis but suggested that, apart from its uricosuric action, the drug might be useful in the control of pain and stiffness associated with chronic gouty arthritis. Of six such cases studied, four were promptly relieved of pain and stiffness with doses of 1.2 gm. daily, two only when the dosage was increased to 2.0 gm. daily. One of the patients, who received the drug in 2.0-gm. daily dosage continuously for 10 months, shows definite reduction in the size of tophi.

Despite relief of chronic joint symptoms, however, G–25671 in 1.0-gm. or 2.0-gm. daily dosage proved to be ineffective as a prophylactic agent in preventing recurrence of acute gouty arthritis in 4 of 7 patients receiving the drug for protracted periods.

Toxic reactions to G–25671

The drug was administered to 28 gouty subjects in all, 21 for studies of less than one week's duration, 7 continuously and without adverse effects for periods of 3 to 10 months. Three patients developed a rash (one urticarial, one erythematous, 1 purpuric), in every instance within a day or two of initiation of medication; one had reacted similarly to phenylbutazone. One patient developed a renal calculus, another noted "sand" in the urine, a third complained of renal colic. One patient complained of anorexia, one of marked constipation, the only gastro-intestinal complications encountered. The drug did not appear to precipitate acute gouty arthritis when first administered but, as already mentioned, did not prevent recurrence of acute seizures in the course of protracted usage. Careful checks at regular intervals in the 7 patients receiving the drug for 3 to 10 months failed to reveal any indication of bone marrow depression, hepatotoxicity or neurotoxicity.

[2] We are indebted to R. Domenjoz and G. Wilhelmi of Geigy Pharmaceuticals for this information.

DISCUSSION

The effects of G–25671 on discrete renal functions, as described in the preceding section, differ in significant respects from those observed with equivalent dosages of phenylbutazone, notably in relation to tubular reabsorption of urate and of salt and water. These differences, brought about by substitution of a phenylthioethyl group for the butyl side chain of the parent compound, appear to be quantitative rather than qualitative in nature, as will be made evident in detailed comparisons to follow. Nevertheless they are of such order of magnitude as to impart potential clinical usefulness to G–25671 as a uricosuric drug in the management of gout (1), phenylbutazone not being serviceable for this purpose (7). The data now at hand make possible a provisional assessment of G–25671 as a uricosuric agent.

Comparison of uricosuric effects of phenylbutazone and G–25671

It was found in a prior study (7) of ten gouty subjects that intravenous injection of phenylbutazone in dosages ranging from 12 to 27 mg. per Kg. body weight caused a rise in C_{urate}/C_{In} ratios from a mean control value of 6.04 per cent to a mean peak of 19.5 per cent, an approximately three-fold increase. The data of the present analogous study with G–25671, given intravenously in a dosage of 16 mg. per Kg. body, indicate an approximately five-fold mean peak rise in C_{urate}/C_{In}, from a premedication value of 6.15 per cent to 30.6 per cent. Direct comparisons, made in the same patient, more convincingly demonstrate the greater uricosuric efficacy of G–25671 (Table I). In Case A. R., the C_{urate}/C_{In} ratio rose from 5.9 per cent to a peak of 39.6 per cent with G–25671 whereas with the same dosage of phenylbutazone the rise was from 5.3 per cent to 21.8 per cent; in Case L. W., the respective increases were from 3.3 per cent to 49.3 per cent with G–25671 and from 3.6 per cent to 17.8 per cent with phenylbutazone; in Case M. C., from 6.1 per cent to 31.5 per cent and from 5.0 per cent to 9.0 per cent, respectively. Since these and similar comparisons of the two drugs were made with dosages equivalent on a drug-weight basis, the actual dosage of G–25671 administered was approximately 25 per cent less than that of phenylbutazone when calculated on an equi-molecular basis (molecular weight of phenylbutazone, 298; of G–25671, 384).[8]

There has been some difference of opinion as to whether phenylbutazone, when administered orally, exerts any uricosuric action in the usual dosage, 400 to 800 mg. daily. The preponderance of evidence (7, 10, 11 and others) clearly indicates that phenylbutazone increases urinary urate excretion when plasma drug concentrations approximating 10 mg. per cent or more are attained, for which regular oral doses of at least 600 mg. per day ordinarily are required (10). A decline in serum urate concentration may precede distinct uricosuria but this appears to be largely factitious, usually the result of hemodilution due to marked salt and water retention.

The available data on G–25671 indicate that this drug, when given orally, elicits increases in urinary urate excretion and associated falls in serum urate concentration at least as great as probenecid in equivalent dosage (12). The uricosuric action begins at plasma drug levels approximating 1 mg. per cent, and effective blood levels are readily achieved when the drug is given orally in the daily dosage we have ordinarily employed, 1.0 gm. per day. Because of the short biologic half-life of G–25671 (about 3 hours compared to 70 hours for phenylbutazone) (1) maintenance of effective plasma drug levels throughout the day requires distribution in divided doses. We give 0.2 gm. every 4 hours from 7:00 A.M. to 11:00 P.M.

Comparison of effects of phenylbutazone and G–25671 on salt and water retention

It is well known that phenylbutazone may cause marked retention of salt and water; indeed, the

[8] Several additional phenylbutazone analogs, representing other substitutions in the side chain, have been tested in respect to their uricosuric action. G–25903, in which a phenylthiopropyl side chain replaces the phenylthioethyl group of G-25671, has much less uricosuric effect than G–25671 or phenylbutazone; 2 gm. per day by mouth elicits only a 25 per cent increase in urinary urate excretion. On the other hand, G–25669 (isopropylthioethyl analog) and G–25921 (p-chlorophenylthioethyl analog) are both very potent uricosuric agents. All three of these compounds undergo rapid biotransformation and have a half-life approximating that of G–25671.

clinical consequences thereof, in particular expansion of the intravascular fluid compartment, represent a hazard in the use of the drug. In our own experience (7), the urinary excretion of sodium in one patient studied, Case O. G., fell from 0.81 gm. per 24 hrs. to 0.37 gm. per 24 hrs. when 0.8 gm. phenylbutazone was infused daily; the corresponding figures for urinary chloride excretion were 1.63 and 0.92 gm. per 24 hrs., respectively. This patient was maintained throughout the experiment on a constant low-sodium diet to which 2.5 gm. sodium chloride a day was added. In another instance (7), Case L. W., who also received 0.8 gm. phenylbutazone by intravenous injection, the urinary excretion of sodium fell from 2.20 to 1.13 gm. per 24 hrs., the urinary chloride excretion concomitantly from 3.61 to 1.80 gm. per 24 hrs. Case L. W.'s low salt diet was supplemented with 3.5 gm. sodium chloride daily. In both instances retention of sodium was associated with a rise in serum sodium levels, in Case O. G. from 141 to 150 mEq. per L, in Case L. W. from 145 to 156 mEq. per L.

These same patients were subjected to analogous experiments with G–25671 which, as indicated by the data of this study already presented, also causes some retention of sodium chloride (Figure 2); in equivalent dosage and plasma drug levels, however, significantly less than does phenylbutazone. Thus the urinary sodium excretion in Case O. G. fell from 1.32 gm. per 24 hrs. to a mean of 0.95 gm. per 24 hrs. for the first two days of intravenous injection of 1.2 gm. G–25671 daily (50 per cent higher dosage than in the corresponding phenylbutazone experiment) but returned to the control level, 1.25 gm. per 24 hrs., as the drug infusion was continued. The corresponding figures for urinary chloride excretion were closely parallel: 1.94 gm. per 24 hrs. control level, 1.47 gm. per 24 hrs. for the first two days, and 2.28 gm. per 24 hrs. for the last two days of injection. The constant low sodium diet in this instance was supplemented with 2.5 gm. sodium chloride daily, as in the corresponding phenylbutazone experiment. Case L. W. received 1.6 gm. G–25671 daily by intravenous injection. At this dosage retention of sodium was about as marked as was obtained with 0.8 gm. of phenylbutazone: The premedication urinary excretion of sodium, 1.69 gm. per 24 hrs., fell to a mean of 0.96 gm. per 24 hrs., the urinary chloride excretion fell from 2.26 to 1.14 gm. per 24 hrs. The low sodium diet in this instance was supplemented with 3.5 gm. sodium chloride, as in the prior phenylbutazone experiment.

Owing to the rapid metabolic degradation of G–25671, the tendency to retain sodium chloride was no longer apparent even on the first day of discontinuance of medication whereas the sodium-retaining effect of phenylbutazone was still in evidence 24 to 72 hours thereafter. G–25671 did not cause any hypernatremia, such as was noted with phenylbutazone, and hemodilution was not significant in ordinary clinical dosage.

Another relevant point in this connection was brought out by slow infusion experiments in which the plasma concentration of phenylbutazone or G–25671 was raised gradually in order to determine the relative drug levels required to effect uricosuria and/or sodium retention (Table II). These experiments demonstrate that plasma phenylbutazone levels of 1 to 2 mg. per cent are associated with distinct sodium retention (Table II) whereas no increase in urinary urate excretion occurs until plasma drug concentrations of approximately 10 mg. per cent are reached (7, 10). On the other hand, G–25671 causes distinct uricosuria at plasma drug concentrations of 1 mg. per cent, which appear to be insufficient to elicit significant sodium retention. In Case L. O., the plasma phenylbutazone level was slowly raised to 8.7 mg. per cent at which point UV_{Na} was decreased 58 per cent and UV_{Cl} declined 66 per cent, yet C_{urate}/C_{In} approximated the pre-medication ratio.[4] Case J. B. exhibited the same phenomenon, although not so markedly, at lower plasma phenylbutazone concentrations. In contrast, C_{urate}/C_{In} increased 130 per cent at a plasma G–25671 concentration of 5.4 mg. per cent in Case L. L., without any decline whatsoever in UV_{Na} or UV_{Cl}. In Case J. S., C_{urate}/C_{In} increased 35 per cent at a plasma G–25671 concentration of 1.3 mg. per cent at which point UV_{Na} and UV_{Cl} were unchanged.

[4] It will be noted that in this and other instances, very low plasma phenylbutazone and G–25671 levels may be associated with *reduction* in C_{urate}/C_{inulin}. The significance of this phenomenon is discussed elsewhere (13).

Comparison of clinical usefulness of phenylbutazone and G–25671 in the management of acute and chronic gouty arthritis

The potent anti-rheumatic properties of phenylbutazone have established a limited but definite place for it in the current management of various arthritic disorders, notably in the short-term treatment of acute gouty arthritis. Our own data in this connection conform to the favorable general experience. Of 103 acute gouty seizures in 61 patients, treatment with 800 mg. phenylbutazone a day gave complete remission within 24 to 48 hours in 76 (74 per cent) and within two days to one week in 14 (14 per cent). Five patients (5 per cent) showed a partial response, three (3 per cent) did not respond at all and five (5 per cent) improved but this was followed by disturbing side effects of the drug. In contrast to these good results with phenylbutazone, only 30 per cent of 10 acute gouty attacks treated with G–25671 (1 gm. daily) remitted completely within 24 to 48 hours, an experience which does not justify use of this drug for termination of acute gouty arthritis. Our experience with G–25671 in protracted daily usage as a prophylactic agent to prevent recurrence of acute gouty seizures also has been discouraging, certainly less satisfactory than with the daily prophylactic use of small doses of colchicine for the purpose (14).

The experience herein described suggests, however, that G–25671 may have potential clinical usefulness as a uricosuric agent in the prevention and treatment of chronic gouty arthritis; it has potent uricosuric and mild anti-rheumatic properties, and causes negligible retention of sodium and water. Since administration as a uricosuric agent would require daily dosage for months or years, the limiting factor in use of the drug for this purpose would appear to be its hazard of significant toxicity. In our own small experience thus far, this hazard does not appear to be excessive. Of 28 gouty subjects treated with the drug, three developed significant side reactions (drug rash, which disappeared promptly upon discontinuance of medication), three had complications referable to excessive uricosuria (a hazard common to all uricosuric agents which can usually be circumvented), none gave evidence of bone marrow depression, hepatotoxicity or neurotoxicity. The similarity in chemical structure to phenylbutazone, however, suggests caution in the protracted administration of G–25671, and much more extensive and prolonged experience will be necessary to permit conclusions as to its safety for use in the management of chronic gouty arthritis.

Phenylbutazone has been recommended for protracted daily use in gouty subjects (15) but we consider that the toxic hazards entailed do not justify its employment for this purpose (7).

SUMMARY

1. G–25671 is a phenylbutazone derivative in which a phenylthioethyl group replaces the butyl side chain. The effects of G–25671 on the simultaneous renal clearances of inulin, para-aminohippurate, urate, sodium, potassium and chloride are reported, and the results compared with those obtained with phenylbutazone.

2. Like phenylbutazone, G–25671 usually does not significantly affect the inulin clearance but may lower it in some instances. Both drugs significantly depress C_{PAH}, apparently as a result of decreased renal extraction of PAH.

3. In seven rapid infusion experiments, G–25671 caused a mean five-fold increase in C_{urate}/C_{In}, from a mean pre-medication value of 6.15 per cent to a mean peak of 30.6 per cent. This uricosuric effect, which is much more pronounced than that of phenylbutazone, is attributable to suppression of tubular reabsorption of urate.

4. G–25671 causes distinctly less sodium and water retention than phenylbutazone in comparable dosage, and exerts its uricosuric action at low plasma drug levels (approximately 1 mg. per cent) which are not associated with any discernible effects on tubular transport of electrolytes. Phenylbutazone, in contrast, causes sodium and water retention at low plasma drug levels and uricosuria only in plasma drug levels of approximately 10 mg. per cent or more. Neither drug significantly affects potassium or phosphate excretion.

5. Administered orally to gouty subjects, in 1.0 to 2.0-gm. daily dosage, G–25671 is a potent uricosuric agent. In 13 such patients, free of significant renal damage, the serum urate level was reduced from a mean pre-medication figure of 9.6 mg. per cent to a mean minimum of 4.7 mg. per cent; the effect in patients with renal damage was

less satisfactory. G–25671 also possesses antirheumatic properties sufficient to control pain and stiffness in chronic gouty arthritis but these are distinctly less pronounced than phenylbutazone and the drug is of little or no value in terminating acute gouty attacks.

6. The limiting factor in clinical use of G–25671 as a uricosuric agent in the prevention and treatment of chronic gouty arthritis is the potential hazard of toxic side reactions. Drug sensitivity (rash) developed in approximately 10 per cent of our patients. There was no indication of bone marrow depression, hepatotoxicity or neurotoxicity in this series but the observations are too few and too brief to warrant conclusions.

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STUDIES ON ALCOHOL DIURESIS. III. THE RESPONSE TO ETHYL ALCOHOL IN CERTAIN DISEASE STATES CHARACTERIZED BY IMPAIRED WATER TOLERANCE [1]

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Ethyl alcohol, when imbibed by normal subjects, stimulates a diuresis of water and blocks the antidiuretic effects of such stimuli as the passive erect posture, infusions of hypertonic saline, venous congestion of the lower limbs, and acetylcholine. It has been shown to inhibit transiently the release of the antidiuretic hormone (ADH) from the posterior pituitary gland, regardless of the nature of the provoking antidiuretic stimulus (1–6). Recently, Dicker (7) has demonstrated an almost complete absence of antidiuretic activity in the blood of rats given lethal amounts of ethyl alcohol. These data afford convincing argument localizing the site of action of this compound to the supraoptico-hypophyseal system.

An excessive activity of ADH has been invoked by many investigators in an attempt to explain the impaired excretion of water which is a feature of certain disease states. In view of its known action in inhibiting the release of this hormone, it was of interest to observe the effect of alcohol when administered to patients with diseases characterized by faulty metabolism of water.

MATERIALS AND METHODS

Observations were made upon 16 hospitalized patients with the following diagnoses: cirrhosis (six), arteriosclerotic, hypertensive, or valvular heart disease with congestive failure (four), cor pulmonale (one), superior vena-caval syndrome (one), nephrotic syndrome (one), Addison's disease (two), and hypoadrenocorticism secondary to panhypopituitarism (one). All but the last three had clinically evident fluid retention (edema, ascites, or both). One Addisonian (R. M.) was receiving cortisone at the time of the study, while the other, and the patient with panhypopituitarism, were not. Impaired water tolerance is defined as failure to excrete, within three to four hours, at least fifty per cent of 1000 to 1500 milliliters of water, administered either orally or intravenously as iso- or hypotonic fructose or glucose, prior to the day of testing.

Ten patients each received a single dose of alcohol (35 to 60 grams) as either 95 per cent laboratory alcohol or 100 proof Bourbon whiskey, made up to a total volume of approximately 150 to 180 cc. with fruit juice and ice. The remaining six patients each received two to four doses of 25 to 45 grams each (total 70 to 140 grams) at intervals of one or two hours.

In an additional study, a single 45-gram dose of alcohol was ingested by two adequately hydrated patients with diabetes insipidus, who were not receiving posterior pituitary therapy at the time.

From 7:00 to 7:30 a.m. on the day of the test, the patient ate a light breakfast including 400 to 600 cc. of fluid. About one hour later, the patient emptied his bladder, and assumed the recumbent or semi-recumbent position, standing only to void. Alcohol was generally given two to three hours afterwards. Water loads of 1000 to 1500 cc. (either by mouth in 15 to 20 minutes, or as 2.5 per cent or 5 per cent glucose or fructose in one hour intravenously) were administered to five patients one and a half to three and a half hours before, and to two patients concurrently with, the ethanol. The other patients drank smaller amounts of water, as indicated in the tables. Each study lasted approximately five to seven hours.

Blood was drawn at approximately hourly intervals for the determination of sodium, creatinine and osmolarity (freezing-point depression) in the serum, and half-hourly for the determination of the concentration of alcohol. Urine, for the determination of sodium, creatinine and osmolarity, was collected in one-half- to two-hourly periods, by spontaneous voiding or indwelling catheter.

The chemical methods and calculations employed have been described in a previous publication (1).

$$C_{osm} = \text{osmolar clearance} = \frac{U_{osm} V}{P_{osm}}$$

$$C_{H_2O} = V - C_{osm}$$

where

U_{osm} = concentration of solute in the urine in mOsm./L.

V = urine flow in cc./min.

P_{osm} = concentration of solute in plasma in mOsm./L.

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RESULTS

For purposes of this study, a diuretic response is defined as the excretion of fifty per cent or more of an administered load of water within four hours after the ingestion of alcohol. For those patients who were not subjected to a provocative water load, a positive response consisted of an increase in urine flow to values significantly greater than those of the control period, a fall in urinary osmolarity and a rise in free water clearance (C_{H_2O}).

I. *Patients with diabetes insipidus, Addison's disease, and panhypopituitarism (Tables I and IIa)*

Alcohol did not further augment the relatively high rates of urinary flow in two patients with diabetes insipidus (Table I).

TABLE I

Response to ethyl alcohol of two patients with diabetes insipidus

Period No.	Elapsed time *min.*	Urinary flow *cc./min.*
	Patient I—J. V.	
1	70	8.0
2	110	11.2
3	158	10.8
4*	213	11.0
5	332	5.6
	Patient II—S. C.	
1	30	6.2
2	60	7.7
3	110	6.7
4	135	10.0
5*	198	4.21
6	240	8.8
7	270	8.3

* Alcohol was administered at the beginning of this period.

In one patient with Addison's disease and one patient with hypopituitarism (patients 1 and 2, Table IIa), neither of whom was receiving cortisone, alcohol did not provoke a water diuresis. Patient 3, R. M., an Addisonian treated with cortisone, who had regained the normal diuretic response to water, showed a similar normal diuretic response following alcohol.

II. *Patients with clinically evident retention of fluid (Table IIb)*

In only four of thirteen patients in this category (patients 4 to 7) was ethanol successful in provoking diuresis. One of these, P. M., also responded normally to ingestion of water, during the control period, as evidenced by urine flows of 4.0 and 3.5 cc. per min in periods 2 and 3, respectively, following a water load given one hour before the beginning of period 2. Imbibing alcohol when the rate of urine flow was falling resulted in a second diuresis. Patient J. L., in whom impaired tolerance to water loading is suggested by flows of 0.4 cc. per min. during periods 1 and 2 (*i.e.*, during the second hour following ingestion of 500 cc. of water), was, however, able to diurese normally upon water loading two weeks later, following paracentesis and salt restriction. The response of patient 6, D. McC., is anomalous. From the temporal relationship between the ingestion of water, ingestion of alcohol, and onset of diuresis, the diuresis seems to be more in response to water than to alcohol. However, on two previous occasions, unequivocal impairment in excretion of a water load had been demonstrated. The least dramatic effect of ethanol is seen in patient A. D., who excreted only 53 per cent of the administered load of water. However, following each of two doses of alcohol, an increased rate of urine flow ensued, unassociated with any change in the clearance of endogenous creatinine.

The remaining nine patients did not have a diuretic response to alcohol, regardless of whether one dose (patients 8 to 13) or several doses (patients 14 to 16) were administered.

In comparing those who responded with a diuresis to alcohol with those who did not, no striking differences were apparent with respect to concentration in the serum and urine of sodium or total solutes, their rates of excretion, or the clearance of endogenous creatinine. Comparable levels of alcohol in the blood were attained in the two groups, and are consistent with values observed in normal subjects receiving equivalent amounts of ethanol (1, 2). Of those responding who received more than one dose of alcohol (patients 5 to 7), some diuretic effect was obtained from the first dose, whereas up to four doses in unresponsive patients failed to provoke an increase in the flow of urine. Patients with impaired water tolerance secondary to cirrhosis and to congestive heart failure were represented in both groups; thus the nature of the underlying disease did not, *per se*, appear to determine the response of any given patient to the diuretic stimulus of alcohol.

TABLE II

Response of patients to ethyl alcohol administration

Patient Diagnosis	Period No.	Period Elapsed time (min.)	Dose of alc. (gm.)	Urine Flow cc./min.	Urine Na mEq./L.	Urine Na μEq./min.	Urine Osm mOsm./L.	Urine Osm μOsm./min.	C_{osm} cc./min.	C_{H_2O} cc./min.	$C_{creat.}$ cc./min.	Serum Na mEq./L.	Serum Osm mOsm./L.	Serum Alc mg. %	Remarks
							IIa Addison's Disease and Hypopituitarism								
1. T. Q.															
Craniopharyngioma with	1	120		1.0	158	158	618	618	2.1	−1.1	103	144	296		1,400 cc. H_2O P.O.
hypopituitarism; no	2†	186	36	1.0	184	184	606	606	2.1	−1.1				16	with alc. 227 cc.
hormonal R_x	3	228		1.0	171	171	661	661	2.3	−1.3	102	140	294	45	(16%) excreted in
	4	319		0.7	147	103	685	480	1.6	−0.9		137	291	12	4 hrs.‡
	5	372		1.0	137	137	548	548	1.9	−0.9					
2. T. P.															
Addison's disease; on	1	165		0.3	158	47	649	195	0.7	−0.4	87	126	265		1,500 cc. 5% fructose infusion
DOCA; no	2	255		*2.9	84	244	289	838	3.2	−0.3					completed ½ hr. before
other hormonal R_x	3†	315	48	*2.3	79	182	274	630	2.5	−0.2					alc. 542 cc. (36%)
	4	375		1.0	119	119	429	429	1.7	−0.7	96				excreted in 4½ hrs.‡
	5	435		0.9	124	112	460	414	1.6	−0.7		120	253	127	
	6	495		0.6	174	104	640	384	1.5	−0.9					
3. R. M.															
Addison's disease; on cortisone and	0	0		—	100	—	658	—	—	—					270 cc. H_2O P.O.
	1	38		1.0	125	125	700	700	2.4	−1.4	98		287		1 hr. before alc.
	2	90		1.5	153	153	658	987	3.5	−2.0			286		737 cc. (273%) ex-
DOCA	3†	139	48	2.8	63	176	276	773	2.7	+0.1				56	creted in 3¾ hrs.§
	4	185		4.7	27	127	122	573	2.0	+2.7	105		286	105	
	5	217		4.1	25	103	164	672	2.4	+1.7			286	48	
		242													
	6	289		2.9	31	89	208	603	2.1	+0.8			286	22	
							IIb Ascites and/or Edema								
4. A. D.															
Nephrotic syndrome;	1	97		1.0	31.3	31.3	272	272	1.0	0.0	34	125	283		1,000 cc. H_2O P.O.
	2	149		1.0			312	312	1.1	−0.1					2 hrs. before alc.
edema and	3†	208	45	1.8	30.6	55.1	220	396	1.4	+0.4			281	28	146 cc. (15%) ex-
hypoproteinemia	4	269		1.1			251	276	1.0	+0.1	26		282	62	creted in those 2
	5†	329	45	2.3	28.8	66.2	169	389	1.4	+0.9			282	85	hrs.; of the "re-
	6	394		1.1			216	237	0.8	+0.3	25	124	283	53	maining" 854 cc.,
															384 cc. (45%) were
															excreted in 4 hrs.‡

* Osmotic diuresis due to infusion of fructose or glucose.
† Alcohol imbibed early in this period.
‡ Impaired diuresis to water load demonstrated previous to test day.
§ Normal diuretic response to water load demonstrated previous to test day.
‖ Normal diuretic response to water load demonstrated several weeks later, after paracentesis.

TABLE II—*Continued*

Patient Diagnosis	Period No.	Period Elapsed time (*min.*)	Dose of alc. (*gm.*)	Urine Flow *cc./min.*	Urine Na *mEq./L.*	Urine Na *μEq./min.*	Urine Osm *mOsm./L.*	Urine Osm *μOsm./min.*	C_{osm} *cc./min.*	C_{H_2O} *cc./min.*	$C_{creat.}$ *cc./min.*	Serum Na *mEq./L.*	Serum Osm *mOsm./L.*	Serum Alc *mg. %*	Remarks
						IIb Ascites and/or Edema—*Continued*									
5. S. H.															
Cirrhosis,	1	57		0.8	12.1	9.7						132			800 cc. H_2O P.O.
ascites, and	2	137		1.0	9.7	9.7					146	135			2 hrs. before alc.
edema	3†	196	35	1.1	9.7	10.7					144			43	233 cc. (29%) ex-
	4	254		0.3	6.2	1.9						134		58	creted in 5 hrs.‡
	5	297		0.6	5.7	3.4						134		51	
6. B. G.															
Cirrhosis,	1	30		0.5	34.7	17.4	678	339	1.4	−0.9	131	115	243		1,200 cc. 2½% glu-
ascites	2	61		0.5	1.6	0.8	726	363	1.5	−1.0		116	243		cose in 1 hr. i.v.
	3	91		*0.7	1.1	0.8	722	505	2.1	−1.4		118	244		completed ¾ hr. be-
	4	151		*0.7	1.0	0.7	731	512	2.1	−1.4	131				fore alc. 129 cc.
	5†	181	60	0.3	1.7	0.5	721	216	0.9	−0.6		112	238	32	(15%) excreted in
	6	241		0.3	2.1	0.6	574	172	0.7	−0.4	91	110	240	40	3 hrs.‡
		271										110	235	76	
	7	333		0.2	4.0	0.8	667	133	0.6	−0.4		111	240	64	
7. M. B.															
Arteriosclerotic	1	63		0.10	13.4	1.3	549	55	0.21	−0.11			244		150 cc. H_2O P.O.
heart disease,	2†	124	35	0.03			488	15	0.06	−0.03			245	36	1½ hrs. before alc.
congestive	3	225		0.05	39.7	2.0	340	17	0.07	−0.02		117	244	47	12 cc. (9%) ex-
heart failure; edema															creted in 3½ hrs.
8. M. A.															
RHD, AI,	0				1.6							143	284		1,000 cc. H_2O P.O.
AS; no	1	50		0.3											with alc. 178 cc.
edema	2†	132	48	0.7	0.7	0.5	535	374	1.3	−0.6			284	190	(18%) excreted in
	3	192		0.6	0.9	0.5	642	385	1.4	−0.8					4 hrs.‡
	4	235		0.6	1.2	0.7	776	466	1.6	−1.0					
	5	257		0.9	1.3	1.2	792	713	2.5	−1.6					
	6	297		0.8	1.0	0.8	797	637	2.2	−1.4					
9. M. P.															
Chronic pul-	1	35		0.7	124	87	374	262	1.0	−0.3	100				1,000 cc. 5% glu-
monary dis-	2	115		*1.6	60	96	378	604	2.4	−0.8		123	253		cose i.v. in 1 hr. be-
ease; cor	3	145		*4.3	26	112	229	985	3.9	+0.4		118	250		tween periods 2–4.
pulmonale;	4	183		*1.7	38	65	246	418	1.7	0.0	65	122	251		430 cc. (43%) ex-
congestive	5†	240	45	0.6	91	55	410	246	1.0	−0.4			256		creted in 3½ hrs.‡
failure;	6	283		0.7	74	52	418	293	1.1	−0.4	68	126			
edema															

TABLE II—*Continued*

Patient Diagnosis	Period No.	Period Elapsed time (min.)	Dose of alc. (gm.)	Urine Flow cc./min.	Urine Na mEq./L.	Urine Na μEq./min.	Urine Osm mOsm./L.	Urine Osm μOsm./min.	C_{osm} cc./min.	C_{H_2O} cc./min.	$C_{creat.}$ cc./min.	Serum Na mEq./L.	Serum Osm mOsm./L.	Serum Alc mg. %	Remarks
							IIb Ascites and/or Edema—*Continued*								
10. S. R.															
Superior	1	70		1.2	24.6	29.5	526	631							1,000 cc. H_2O P.O.
vena-caval	2	109		1.2			526	631							2½ hrs. before alc.
syndrome	3	145		1.3			481	626							370 cc. (37%) ex-
	4	180		1.9			310	589							creted in 5 hrs.‡
	5	211		1.3			473	615							
	6†	240	45	0.7			638	446							
	7	270		0.9			704	642							
	8	340		0.6			721	433							
11. C. M.															
Cirrhosis	1	49		0.6	3.3	2.0	329	198	0.7	−0.1	71	128	267		700 cc. H_2O P.O.
with ascites	2	93		1.0	1.7	1.7	232	232	0.9	+0.1		124	263		2½ hrs. before alc.
and edema	3†	130	35	0.5	2.7	1.4	296	148	0.6	−0.1	66			70	171 cc. (24%) ex-
	4	165		0.4			449	180	0.7	−0.3				59	creted in 6½ hrs.‡
	5†	228	35	0.3	11.6	3.5	459	138	0.5	−0.2		127	266		
		258												115	
		288												145	
	6†	346	35	0.3	6.0	1.8	432	130	0.5	−0.2	61			143	
	7	413		0.5	2.4	1.2	284	142	0.5	0.0		128	277	142	
12. P. M.															
Cirrhosis	0	0			4.4		348					139	278		600 cc. H_2O P.O.
with ascites	1	70		1.6	1.6	2.6	161	258	0.9	0.7					2½ hrs. before alc.
±edema	2	141		4.0	1.3	5.2	81	324	1.2	2.8	106				435 cc. (72%) ex-
	3	157		3.5	2.7	9.4	78	273	1.0	2.5			274		creted before alc.;
	4†	220	35	1.3	3.3	4.3	106	138	0.5	0.8				74	of the "remaining"
	5	275		3.0	1.3	3.9	100	300	1.1	1.9	111	138	274	92	165 cc., 278 cc.
	6	336		0.7	1.2	0.8	156	109	0.4	0.3		140	277	46	(169%) were ex-
				0.7											creted in 2¾ hrs.
															after alc.
13. J. L.															
Cirrhosis	1	30		0.4	11.3	4.5	661	264	1.0	−0.6		133	274		500 cc. H_2O P.O.
with ascites	2	60		0.4	2.7	1.1	688	275	1.0	−0.6	101				2 hrs. before alc.;
and edema	3†	97	35	0.5	2.0	1.0	659	330	1.2	−0.7		132	273		25 cc. (5%) ex-
	4	127		2.6	0.6	1.6	177	460	1.7	+0.9	112			33	creted in those 2
	5	159		1.1	1.7	1.9	231	254	0.9	+0.2		132	274	30	hrs.; of the "re-
	6†	191	35	0.5	3.3	1.7	541	271	1.0	−0.5				68	maining" 475 cc.,
	7	231		3.1	1.1	3.4	159	493	1.8	+1.3	112	135	279	78	404 cc. (85%) were
	8	272		3.1	0.7	2.2	104	322	1.2	+1.9		132	273	96	excreted in 4¼ hrs.
	9	317		0.2	2.5	0.5	473	95	0.3	−0.1		130	273	66	after alc.‖

TABLE II—*Continued*

Patient Diagnosis	Period No.	Period Elapsed time (*min.*)	Dose of alc. (*gm.*)	Urine Flow *cc./min.*	Urine Na *mEq./L.*	Urine Na *μEq./min.*	Urine Osm *mOsm./L.*	Urine Osm *μOsm./min.*	C_{osm} *cc./min.*	C_{H_2O} *cc./min.*	$C_{creat.}$ *cc./min.*	Serum Na *mEq./L.*	Serum Osm *mOsm./L.*	Serum Alc *mg. %*	Remarks
							IIb Ascites and/or Edema—*Continued*								
14. D. McC.															
Hypertensive	1	62		0.7	6.3	4.4	306	214	0.7	0.0		144	289		800 cc. H_2O P.O.
cardiovascu-	2	106		2.5			169	439	1.6	1.0	60				1 hr. before alc.
lar disease;	3†	131	35	10.0			71	721	2.6	7.4		125	280	32	1,096 cc. (137%)
congestive	4	150		8.9			71	631	2.2	6.7	71				excreted in 5¼ hrs.‡
heart failure;	5	171		5.7			71	405	1.4	4.3		130	281	46	
edema	6†	217	35	3.0			125	375	1.3	1.7				69	
	7	245		2.0			155	310	1.1	0.9	58	125	281	83	
	8†	305												113	
		401	35	1.7			165	281	1.0	0.7		133	282	129	
	9	438		1.0			211	211	0.7	0.3	51	138	283	83	
15. V. P.															
Cirrhosis	0†	0	35				500					131	268		600 cc. H_2O P.O.
with ascites	1	57		0.4	3.6	1.4	445	178	0.7	−0.3	36				1 hr. before alc.
and edema	2†	121	35	0.5			455	228	0.8	−0.3	58			111	400 cc. H_2O P.O.
	3†	180	35	0.7			337	232	0.9	−0.2	51		277	145	10 min. before alc.
		249												118	232 cc. (23%) ex-
		329												134	creted in 7 hrs.‡
	4†	360	35	0.7			275	193	0.7	0.0	48		268	173	
16. L. J.															
RHD, with	0	0			0.7		308					136			800 cc. H_2O P.O.
A.S., M.I.	1	75		1.0	0.4	0.4	221	221	0.8	0.2	61				3 hrs. before alc.
+M.S.,	2	120		1.1	0.5	0.6	185	204	0.8	0.3		130	264	10	320 cc. (40%) ex-
CHF; ascites	3†	167	25	0.7	0.6	0.4	224	157	0.6	0.1					creted in 8 hrs.‡
and edema	4	212		1.6	0.3	0.5	179	286	1.1	0.5	77		264	28	
	5†	243	45	0.2	0.6	0.1	292	58	0.2	0.0				92	
		308												124	
	6	390		0.5	0.7	0.4	265	133	0.5	0.0	60		265	110	
		482											264	184	
	7†	501	35	0.2	0.6	0.1	289	58	0.2	0.0			266	147	

DISCUSSION

That ethanol caused no further increase in the urine flow of patients with diabetes insipidus is not surprising. If the action of alcohol is indeed inhibition of the release of ADH, then when the release of this hormone is already minimal or absent, owing to damage to the neurohypophysis, further suppression could not reasonably occur. This observation merely helps affirm earlier argument localizing the site and mechanism of alcohol diuresis.

Because of the known action of the antidiuretic hormone in inhibiting the excretion of water, attempts have been made to relate the impaired water metabolism of various clinical conditions, with and without edema, to over-production of ADH (8), diminished destruction of ADH, or increased sensitivity to this hormone (9). In support of these hypotheses are the reported findings of an elevated amount of antidiuretic substances in the urine or blood of patients with such conditions. Elevated values have been reported in cirrhosis (10–12), congestive heart failure (13, 14), hypertension (15), and Addison's disease (11). However, these substances are not the same in all instances, nor have they been shown conclusively to be identical with ADH (13), Perry and Fyles (16) found no correlation between levels of an antidiuretic substance and edema or liver damage, and no significant difference in antidiuretic activity between the sera of normals and patients with congestive failure or cirrhosis. Nelson and Welt (17) concluded that the patient with cirrhosis and ascites showed no evidence of an increased sensitivity to, or an inability to eliminate or inactivate, endogenous or exogenous posterior pituitary hormone. van Dyke, Ames, and Plough (18), and Stein, Schwartz, and Mirsky (19) report that despite increased levels of antidiuretic substance in some patients, no correlation between this and impaired water metabolism could be detected. van Dyke (20) has justly criticized much of the methodology which has been employed in the assay of antidiuretic substances.

Gaunt (21), working with animals, and Lloyd and Lobotsky (9, 11) on the basis of human studies, have postulated an antagonistic relationship between the adrenal cortex and the posterior pituitary with respect to the control of the balance of water in the body. Thus, in the adrenalectomized rat or the adrenal-insufficient human, an increased amount of ADH may accumulate in the blood, preventing excretion of a water load in normal fashion. The administration of corticosteroids, according to this theory, by antagonizing the action of ADH (or of some factor which promotes the release of ADH), permits normal water diuresis to ensue. However, if the action of the corticosteroids is in fact the inhibition of ADH, one should expect similar results from alcohol. In the present studies, a patient with Addison's disease (T. P.) and a patient with panhypopituitarism (T. Q.), neither of whom was receiving corticoid therapy, both failed to have a diuresis after ingesting alcohol. The administration of cortisone, however, which is essential for the correction of the abnormal water tolerance in these states, to another Addisonian (R. M.) also restored the responsiveness to alcohol. One may conclude, therefore, that the action of cortisone is probably not antagonistic to ADH, but is rather "permissive" (22), allowing the organism, by mechanisms as yet undefined, to respond in normal fashion to the stimuli of both water and alcohol.

The inhibitory effect of alcohol on the neurohypophysis is transient. It appears to be related to the duration of the rise in the level of alcohol in the blood, rather than to the absolute level attained (4). Therefore, the administration of repeated doses of ethanol, when the blood level is falling, should theoretically prolong the inhibition established by the initial dose, and allow for a more leisurely dissipation of circulating ADH. A response to a second dose, in the face of minimal or absent response to the first, would suggest either that the destruction of ADH is impaired, or that an increased amount of this hormone was being secreted by the posterior pituitary into the blood. However, in patients who were unresponsive, up to four doses of alcohol failed to provoke diuresis, whereas in those who did respond to multiple doses, some increase in urine flow followed the ingestion of the initial dose.

Of the sixteen patients observed in the present study, all but three (R. M., P. M., and J. L.) had unequivocally impaired responses to water. Since ethyl alcohol, a substance known to inhibit the release of ADH from the neurohypophysis, failed to provoke diuresis in 11 of 13 patients with abnormal water tolerance, the present data do not

support the hypothesis that excessive activity of the antidiuretic hormone of the posterior pituitary is the most important causative factor in the impaired water metabolism of the diseases studied. Although some activity of ADH cannot be excluded, other factors, the nature of which remain unknown, may well play the dominant role in the pathogenesis of the impaired water tolerance that characterizes these states. Whatever these water-retaining stimuli might be, it is probable that the intensity of their action varies from individual to individual, regardless of the nature of the disease. Should this be true, the extent of water diuresis in the absence of ADH (or in response to alcohol) would be determined by the intensity of action of these extra-neurohypophyseal factors.

SUMMARY

1. Ethyl alcohol, a known inhibitor of the release of ADH from the neurohypophysis, was administered orally to 16 patients with various diseases characterized by impaired water tolerance.

2. Eleven of thirteen patients in whom impaired water tolerance was demonstrated failed to respond to ethanol with a diuresis of water.

3. Cortisone restored the ability of one patient with Addison's disease to respond normally with a diuresis both to water and to alcohol.

4. These data do not support the contention that excessive activity of ADH is chiefly responsible for the disturbed water tolerance characterizing the disease states studied.

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THE PHYSICAL PROPERTIES OF THE LUNG IN CHRONIC OBSTRUCTIVE PULMONARY EMPHYSEMA [1]

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The physical properties of the lung with which this paper is concerned, are the forces which are required to overcome the resistances in the respiratory system. These resistances are the elastic resistance of the lung to expansion, and the various viscous resistances involved in the movement of air into and out of the lungs with associated movement of lung tissue.

Simultaneous recording of air flow and intrathoracic pressure enables assessment of the forces required to overcome these resistances. The elastic resistance of the lungs is expressed as the compliance (volume change in liters per centimeter of water-pressure change from end expiration to end inspiration), while the viscous resistance can be differentiated into that proportional to the velocity of air flow (streamline resistance in the airways and movement of lung tissue) and that proportional to the square of the velocity of air flow (turbulent resistance in the airways) (1).

This paper reports the disturbances in the physical properties of the lungs in patients with chronic pulmonary emphysema and in a group of emphysematous patients who had associated right heart failure.

SUBJECTS AND METHODS

Subjects consisted of seven normal individuals (Table I) and 18 patients with chronic obstructive airway disease of mild to moderate degree, six of whom had developed right heart failure (Tables II and III). All gave a history of dyspnea on exertion for two to twenty years. The diagnosis was based on the evidence of disturbances of pulmonary function (Tables II and III) and the clinical findings of an increased antero-posterior diameter of the chest, depressed diaphragms with limitation of movement, and signs of bronchial obstruction on physical examination. Although not shown in the tables, the shape of the expiratory vital capacity tracing was characteristic of obstruction to expiratory outflow in all cases. Right heart failure was diagnosed on the basis of electrocardiographic findings of right ventricular hypertrophy and the clinical findings of venous hypertension, enlarged liver and peripheral edema.

Lung volumes and maximal breathing capacities were predicted and measured by methods described by Baldwin, Cournand, and Richards (2). Dead space/tidal air ratios, venous admixture and maximal diffusing capacities were determined by methods described by Riley and his associates (3, 4).

Esophageal pressure was used to estimate intrathoracic pressure (5–7) in spite of known differences between esophageal and intrapleural pressures (8), because values for compliance and resistance determined with esophageal pressures approximated those using intrapleural pressure sufficiently in the tidal volume range for evaluation of the physiologic abnormalities under consideration (9). The esophageal pressure was measured by means of an air filled, thin-walled rubber balloon which has been previously described (8). All measurements were made within the lower third of the esophagus.

The rate of air flow into and out of the lungs was measured by means of a pneumotachometer containing a 325 mesh stainless steel screen which offered a resistance of 0.07 mm. H_2O per liter per minute of air flow. The pressure drop across the pneumotachometer screen was measured by means of a Statham differential pressure strain gauge transducer (maximum range ± 0.05 p.s.i.). The volume of air breathed was determined by measuring the area under the pneumotachograph with a planimeter.

Subjects breathed through a mouth piece connected to the pneumotachometer by a short length of rubber tubing, one inch in diameter. A solenoid-operated slide valve was introduced between the mouth of the subject and the pneumotachometer in order to interrupt the flow of air at the mouth at varying times during the respiratory cycle. A side tap in the tubing between the subject's mouth permitted the esophageal-mouth differential pressure to be measured by means of a Statham differ-

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TABLE I

Physical properties of the lung in normal subjects

Subject	Age (*yrs.*)	Sex	Height (*cm.*)	VC (*liters*)	Compliance (*l./cm. H_2O*)		Coeff. of linear resistance	Coeff. of turbulent resistance	Work of breathing		
					Functional	Static			Elastic	Viscous (*gm. m./liter vent.*)	Total
G. B.	32	M	178	5.56	.212	.202	1.7	0.13	14.0	21.4	23.0
R. C.	30	M	179	5.92	.245	.248	1.2	0.16	14.5	4.9	16.9
H. D.	34	M	174	4.86	.295	.333	1.5	0.38	28.2	8.2	32.0
J. F.	22	M	180	5.20	.226	.222	1.6	0.26	18.5	20.0	24.9
J. K.	25	F	170	3.82	.142	.133	1.8	0.24	20.0	17.9	29.5
V. N.	24	F	163	3.64	.120	.121	3.0	0.12	24.0	15.2	31.5
R. R.	42	M	177	5.24	.140	.148	1.2	0.30	17.5	19.0	23.0
Mean					.197	.201	1.71	0.23	19.4	15.2	25.8

ential pressure strain gauge transducer (maximum range ± 0.6 p.s.i.).

Tracings of the air flow and esophageal-mouth differential pressure were recorded simultaneously by means of a Sanborn amplifying recorder.

All subjects were studied in the sitting position. The subjects with pulmonary emphysema were studied prior to, and 10 to 15 minutes after, the inhalation of nebulized bronchodilator (Isuprel®). The pneumotachometer was calibrated using rotameters and the pressure gauges were calibrated using a water manometer after each study.

Simultaneous measurements of the pressure and volume changes from end-expiration, determined at intervals of 0.2 seconds, were plotted one against the other during a complete breathing cycle. The area of the loop which was obtained represented the mechanical work done on the lungs in order to overcome viscous resistance, and was expressed in gram meters.

The pressure required to overcome the elastic resistance of the lungs during the breathing cycle was determined by joining the points at end expiration and end inspiration. The elastic properties of the lungs were expressed as the volume change per centimeter of water pressure change or the compliance. The mechanical work required to overcome elastic resistance was determined by the method described by McIlroy, Marshall, and Christie (10) and Comroe, Forster, DuBois, Briscoe, and Carlsen (11), and expressed in gram meters.

It may be noted that there is not complete agreement regarding the method of measurement of the elastic work done on the lung. Otis (12) has described another method but because esophageal pressure does not yield absolute intrathoracic pressure (8), it was not possible to estimate elastic work by this method.

In the normal subjects, the expiratory portion of the pressure-volume loop usually fell within the elastic work

TABLE II

Estimation of ventilatory function in patients with pulmonary emphysema

Group	Subject	Age (*yrs.*)	Sex	Ht. (*cm.*)	History of dyspnea (*years*)	Vital capacity (*liters*)			Maximum breathing capacity (*l./min.*)		
						Predicted	Before Bronchodilatation	After Bronchodilatation	Predicted	Before Bronchodilatation	After Bronchodilatation
I	E. B.	48	M	189	6	4.00	3.27	3.43	111	45	56
	G. B.	46	M	174	2	3.90	2.63	2.79	110	32	37
	S. F.	54	M	178	2	3.90	2.84	2.97	104	80	86
	W. H.	60	M	168	6	3.42	1.78	2.14	91	38	40
	N. J.	33	F	160	10	2.90	1.74	2.05	89	25	34
	H. L.	53	M	161	3	3.34	1.80	2.25	92	22	28
	A. P.	52	M	171	6	3.95	4.17	4.17	100	68	79
	F. S.	46	M	173	15	3.83	3.47	4.33	98	45	54
	W. S.	67	M	175	20	3.50	2.57	2.57	90	32	43
	C. T.	62	M	174	3	3.60	2.81	3.11	92	38	57
	B. W.	52	M	172	3	3.70	2.55	3.06	100	45	51
	C. W.	33	F	145	5	2.45	2.60	3.10	75	26	33
II	F. B.	64	F	153	15	2.25	1.19	1.87	69	11	20
	W. B.	58	M	165	20	3.32	2.13	2.62	89	59	64
	E. H.	42	M	160	13	3.60	2.66	3.54	100	40	51
	J. M.	42	M	173	20	3.90	2.95	3.40	112	34	37
	L. S.	45	F	150	10	2.40	0.83	1.28	70	17	25
	E. W.	54	F	151	20	2.28	1.28	1.73	79	11	23

TABLE III

Estimation of distribution and diffusion in patients with pulmonary emphysema

Group	Subject	Arterial P_{O_2}	Arterial $P_{\bar{v}}$	(1) R.V./T.L.V. ×100	(2) VD/VT ×100	(3) $\dot{Q}VA/\dot{Q}T$ ×100	(4)† DO_2 (*ml./min./mm. Hg*) Pred.	(4)† DO_2 (*ml./min./mm. Hg*) Obs.
I	E. B.	37	61	52	46	13	58	13
	G. B.	45	78	64	48	4	51	10
	S. F.	38	80	44	22	9	48	43
	W. H.	46	83	—	51	4	39	19
	N. J.	43	88	—	41	12	45	13
	H. L.	52	57	—	44	18	37	22
	A. P.	—	—	—	—	—	—	—
	F. S.	40	80	—	49	8	49	25
	W. S.	38	75	58	42	7	40	31
	C. T.	40	85	—	46	7	41	31
	B. W.	42	59	—	49	20	44	18
	C. W.	38	86	—	—	—	—	—
II	F. B.	70	40	—	58	17	20	6*
	W. B.	54	78	55	51	6	37	18
	E. H.	60	46	—	41	6	48	23
	J. M.	58	57	—	50	20	51	20
	L. S.	54	70	—	50	13	27	10*
	E. W.	60	49	—	65	30	23	9

(1) Ratio of residual volume to total lung volume.
(2) Ratio of dead space to tidal volume.
(3) Ratio of venous admixture to total blood flow.
(4) Maximal diffusing capacity for oxygen.
Group I—patients with emphysema.
Group II—patients with emphysema and heart failure.
* Studied at rest.
† Data obtained from Shepard and his associates (reference 22).

area during quiet breathing, indicating that expiratory viscous resistance was overcome by stored elastic energy. Any portion of the pressure-volume loop falling outside the elastic work area represented the additional active work required for expiration. The total work done on the lungs was calculated by adding the elastic work, the inspiratory viscous work and additional expiratory viscous work. The work per liter of ventilation was obtained by dividing the work per respiration by the volume of breath in liters.

The pressure required to overcome viscous resistance was determined from the pressure-volume loop or by interrupting the flow of air at the mouth with the solenoid interrupter. In the former case this was done

TABLE IV

Physical properties of the lung in patients with pulmonary emphysema

Subject	Compliance (*l./cm. H_2O*) Functional Before Bronchodilatation	Compliance (*l./cm. H_2O*) Functional After Bronchodilatation	Compliance (*l./cm. H_2O*) Static	Coeff. of linear resistance Before Bronchodilatation	Coeff. of linear resistance After Bronchodilatation	Coeff. of turbulent resistance Before Bronchodilatation	Coeff. of turbulent resistance After Bronchodilatation
E. B.	0.311	0.354	0.444	3.0	2.5	1.4	0.8
G. B.	0.210	0.280	0.358	2.4	2.2	0.4	0.3
S. F.	0.155	0.176	0.250	3.3	2.3	0.1	0.1
W. H.	0.303	0.360	0.450	2.8	2.5	1.0	0.8
N. J.	0.181	0.206	0.530	2.2	2.0	0.3	0.2
H. L.	0.088	0.211	0.240	3.8	3.1	0.4	0.2
A. P.	0.210	0.242	0.277	5.0	3.1	2.4	0.3
F. S.	0.214	0.246	0.480	2.5	1.3	1.4	1.1
W. S.	0.120	0.155	0.212	10.8	5.0	1.1	0.2
C. T.	0.200	0.223	0.333	2.1	0.9	1.7	0.8
B. W.	0.250	0.325	0.600	3.3	2.4	1.7	0.3
C. W.	0.140	0.312	0.329	3.9	1.9	0.5	0.1
Mean	0.198	0.257	0.379	3.76	2.43	1.03	0.43

TABLE V

Physical properties of the lung in patients with pulmonary emphysema and right heart failure

Subject	Compliance (l./cm. H_2O) Functional Before Bronchodilatation	Functional After Bronchodilatation	Static	Coeff. of linear resistance Before Bronchodilatation	Coeff. of linear resistance After Bronchodilatation	Coeff. of turbulent resistance Before Bronchodilatation	Coeff. of turbulent resistance After Bronchodilatation
F. B.	0.082	0.102	0.183	8.0	4.1	1.1	0.4
W. B.	0.074	0.099	0.185	1.5	1.2	1.7	1.0
E. H.	0.035	0.040	0.103	6.6	6.0	1.5	0.8
J. M.	0.033	0.063	0.190	10.7	5.4	9.8	6.0
L. S.	0.030	0.042	0.072	6.0	1.2	1.8	0.8
E. W.	0.044	0.056	0.105	3.9	2.8	1.4	0.3
Mean	0.050	0.067	0.140	6.11	3.45	2.88	1.5

by subtracting the pressure required to overcome elastic resistance from the intrathoracic pressure for any particular instant in the respiratory cycle. In the latter case the difference between the intrathoracic pressure, measured the instant prior to interruption, and the pressure measured during interruption, yielded the pressure required to overcome air and tissue viscous resistance. These pressures were plotted against simultaneously recorded rates of air flow. Values for the coefficients of linear and turbulent resistance to air flow during inspiration were approximated from these pressure-flow curves by determining the first and second derivatives as described by Fry, Ebert, Stead, and Brown (13).

RESULTS

The physical properties of the lungs found in the normal individuals are presented in Table I. The results obtained before and after bronchodilatation in patients with pulmonary emphysema are presented in Table IV, while those obtained in the emphysematous patients who had developed right heart failure are presented in Table V.

A. Compliance

In patients with pulmonary emphysema it was noted that the compliance of the lungs varied with the rate at which the subject was breathing. The change in compliance during slow breathing could not be attributed to changes in resting level of the lung since there was no change in lung volume as determined on a spirometer tracing, when inspiration was slowed. In order to determine the effect of respiratory rate, the compliance of the lungs was determined during inspirations of approximately equal volume but varying in duration.

The compliances found with the different durations of inspiration in a representative subject from each of the three groups are shown in Figure 1. Each point represents the average of three to six determinations. It can be seen that in the patients with pulmonary emphysema the compliance increased considerably when inspiration was slowed, until a plateau was reached after which it remained fairly constant. The compliance obtained while breathing at the resting respiratory rate is hereafter referred to as the functional com-

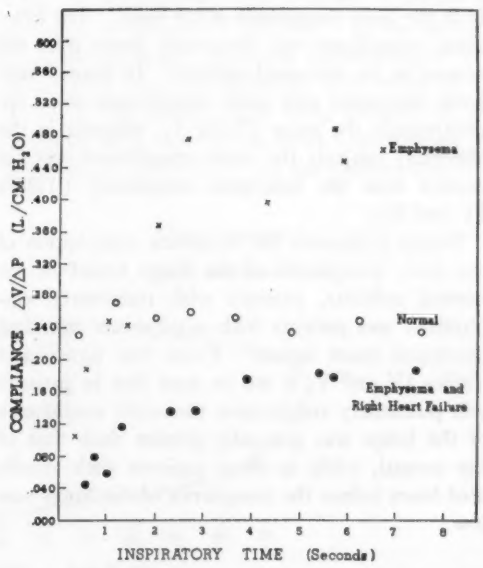

FIG. 1. THE EFFECT OF VARYING DURATION OF INSPIRATION ON COMPLIANCE OF THE LUNG IN A NORMAL SUBJECT, A SUBJECT WITH EMPHYSEMA, AND A SUBJECT WITH EMPHYSEMA AND RIGHT HEART FAILURE

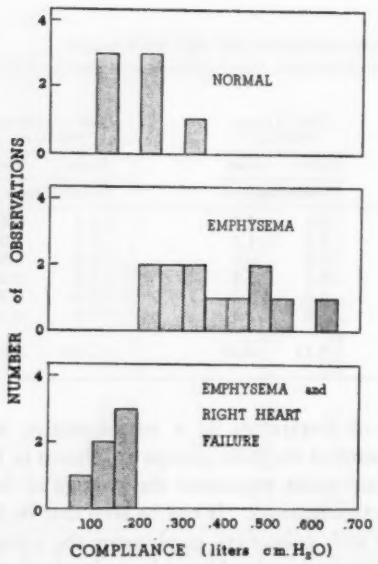

FIG. 2. FREQUENCY DISTRIBUTION OF STATIC COMPLIANCE IN NORMALS, EMPHYSEMA, AND EMPHYSEMA WITH RIGHT HEART FAILURE

pliance, while the value for compliance at the plateau, during the slow inspirations, is referred to as the static compliance of the lungs. The functional compliance was frequently lower than the normal in the abnormal subjects. In normal subjects, functional and static compliances were approximately the same (Table I), whereas in the abnormal subjects the static compliance was far greater than the functional compliance (Tables IV and V).

Figure 2 presents the frequency distribution of the static compliance of the lungs found in the normal subjects, patients with pulmonary emphysema and patients with emphysema who had developed heart failure. From this figure and Tables IV and V, it can be seen that in patients wih pulmonary emphysema the static compliance of the lungs was generally greater than that of the normal, while in those patients with associated heart failure the compliance of the lungs was low.

B. Viscous resistance

The coefficients of linear and turbulent resistance found in the normals are presented in Table I, while those found in the patients with pulmonary emphysema are presented in Tables IV and V. The mean coefficient of linear resistance during spontaneous respiration was lowest in the normal subjects, and highest in those patients who had developed right heart failure, being 1.71 in the normals, 3.76 in the group with emphysema, and 6.11 in the group which had developed heart failure. Similar results were found for the mean coefficients of turbulent resistance, being 0.23 in the normal subjects, 1.03 in the emphysematous patients and 2.88 in the patients in whom heart failure was also present.

C. Work of breathing

Frequency distributions of the work of breathing at the normal resting respiratory rate in the normal subjects, patients with pulmonary emphysema and patients with emphysema who had developed right heart failure are presented in Figure 3. It can be seen that the work required to overcome elastic resistance was slightly higher than the normal in the emphysematous subjects and much higher in the patients with associated right heart failure. The total work of breathing was far greater than the normal in the emphysematous patients and still greater in the group who had developed right heart failure, this increase being particularly due to that required to overcome viscous resistance. In the abnormal subjects the expiratory portion of the pressure-volume loop frequently fell outside the elastic work area indicating active work during expiration.

D. Effect of nebulized bronchodilator

The functional compliance noted during quiet breathing after the administration of bronchodilator was frequently greater than that observed before bronchodilator (Tables IV and V). Although not shown in the tables, the inhalation of bronchodilator did not alter the static compliance since a plateau was reached at the same level that was obtained prior to the administration of bronchodilator.

An example of the effect of nebulized bronchodilator in a patient with emphysema and right heart failure is illustrated in Figures 4 and 5. It can be seen that inhalation of the bronchodilator

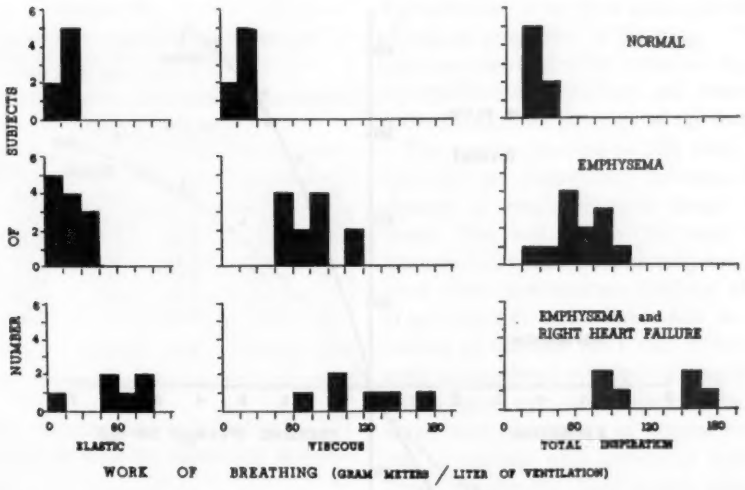

FIG. 3. FREQUENCY DISTRIBUTION OF WORK REQUIRED TO OVERCOME ELASTIC RESISTANCE, VISCOUS RESISTANCE AND TOTAL WORK OF BREATHING IN NORMALS, EMPHYSEMA, AND EMPHYSEMA WITH RIGHT HEART FAILURE

resulted in a considerable reduction in elastic and viscous resistance and work required to overcome these resistances.

The effect of nebulized bronchodilator on the viscous resistance in the abnormal subjects is presented in Tables IV and V. In the emphysematous subjects there was a 35 per cent decrease in the mean co-efficient of linear resistance, and a 58 per cent decrease in the mean co-efficient of turbulent resistance. In the emphysematous subjects who had developed right heart failure, the mean co-efficient of linear resistance diminished by 43 per cent, while the mean co-efficient of turbulent resistance diminished 48 per cent.

The effect of nebulized bronchodilator on the work of breathing in the abnormal subjects is presented in Tables VI and VII. In the emphysematous patients, nebulized bronchodilator resulted in a mean reduction in total work of 17 per cent, the elastic work being reduced by 24 per cent, while the viscous work was reduced by 26 per cent (Table VI). In the patients who had

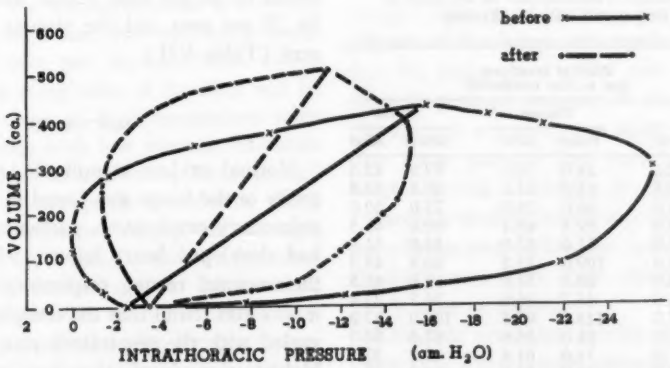

FIG. 4. THE EFFECT OF NEBULIZED BRONCHODILATOR ON THE PRESSURE-VOLUME LOOP IN A SUBJECT WITH EMPHYSEMA AND RIGHT HEART FAILURE

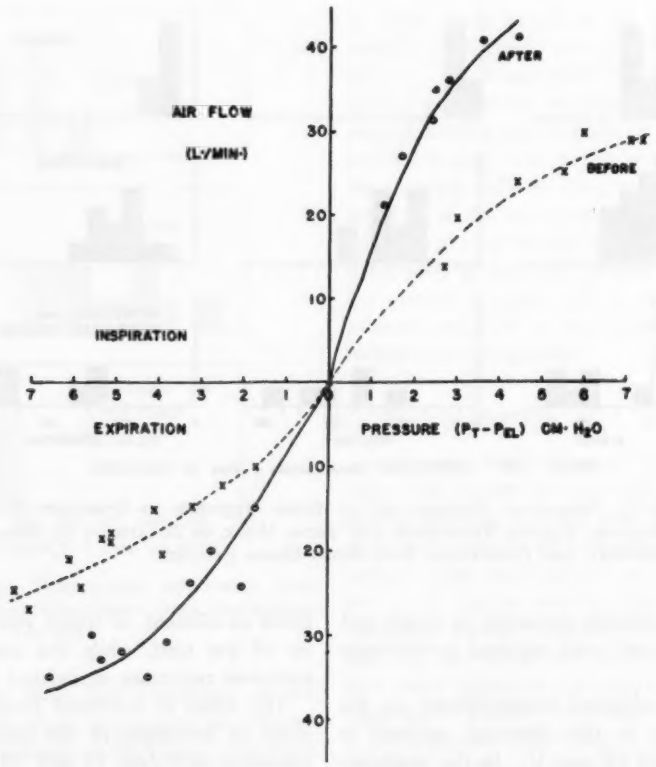

FIG. 5. THE EFFECT OF NEBULIZED BRONCHODILATOR ON PRESSURE-FLOW RELATIONSHIPS IN A SUBJECT WITH EMPHYSEMA AND RIGHT HEART FAILURE

TABLE VI

The effect of nebulized bronchodilator on the work of breathing in patients with emphysema

	Work of breathing (*gm. m./liter ventilation*)					
	Elastic		Viscous		Total	
Subject	Before	After	Before	After	Before	After
E. B.	14.0	12.6	54.0	38.6	52.5	42.6
G. B.	13.5	10.5	84.5	63.6	59.8	53.8
S. F.	37.0	30.0	46.0	28.0	75.0	40.0
W. H.	15.1	13.5	59.5	46.4	60.6	46.5
N. J.	19.6	19.0	71.6	57.0	52.0	51.9
H. L.	29.0	13.0	109.0	75.5	80.8	49.3
A. P.	21.0	16.0	80.2	57.9	54.0	41.5
F. S.	9.0	8.0	45.7	26.6	26.2	23.4
W. S.	25.0	17.0	115.0	93.5	102.0	83.0
C. T.	36.0	29.0	88.0	58.6	87.6	50.9
B. W.	15.0	11.5	71.0	61.4	43.2	37.3
C. W.	31.0	22.0	88.0	65.0	66.3	25.1
Mean	22.2	16.8	76.0	56.0	55.0	45.4

developed right heart failure total work was reduced by 28 per cent, elastic work being reduced by 28 per cent and the viscous work by 20 per cent (Table VII).

DISCUSSION

Normal or low compliance, *i.e.*, increased rigidity of the lungs was found in the patients with pulmonary emphysema, particularly in those who had developed heart failure, while breathing at their normal resting respiratory rate. However, it was also found that the compliance of the lungs varied with the respiratory rate, becoming much higher when inspiration was particularly slow. This change is consistent with the findings of Otis and McKerrow (14) in a lung model, and

TABLE VII

The effect of nebulized bronchodilator on the work of breathing in patients with emphysema and right heart failure

	Work of breathing (gm. m./liter ventilation)					
	Elastic		Viscous		Total	
Subject	Before	After	Before	After	Before	After
F. B.	15.0	12.5	91.0	73.8	78.7	68.8
W. B.	50.3	40.0	72.2	48.0	77.3	70.2
E. H.	88.0	70.5	130.0	95.8	165.8	137.5
J. M.	63.0	40.6	140.4	79.8	182.0	95.4
L. S.	85.0	66.0	170.2	95.0	175.7	113.3
E. W.	55.0	40.0	94.6	68.5	96.2	70.4
Mean	59.4	44.9	116.4	76.8	129.3	92.6

Mead, McIlroy, Selverstone, and Radford (15) in normal individuals after the inhalation of nebulized histamine.

In the basic work on this subject, Bayliss and Robertson (16) and Dean and Visscher (17) found an increased rigidity of the lungs with increased respiratory rate. They explained these findings by visualizing the lungs as a system with two elastances, only one of which was ventilated at rapid respiratory rates while both took part in ventilation at lower rates. Such a hypothesis also serves to explain the changes in compliance with respiratory rate in the patients reported in this study. In these patients the changes in estimated compliance at different respiratory rates are most readily explained by the effects of a nonuniformly distributed viscous resistance. A similar explanation has also been suggested by Mead, Lindgren, and Gaensler (18). When inspiration is slowed so that the viscous element is virtually eliminated, both high and low resistance areas of the lung will take part in ventilation and a high value for the compliance of the lung will be obtained. Conversely, at high respiratory rates portions of the lung with low viscous resistance will be overdistended and the compliance derived will be low. The change in compliance noted after the viscous resistance of the air passages had been reduced by the inhalation of a bronchodilator drug would further support this concept. For these reasons the term functional compliance, which is subject to variation with the respiratory rate, has been used to denote the elastic resistance offered by the lungs while breathing, in contrast to the static compliance which is independent of the resistance to air flow and represents the overall elastic properties of the lung. The latter can only be measured when sufficient time is allowed for equilibrium of volume and pressure to occur between different portions of the lung.

The results reported in this study may help to reconcile the discrepancy between the increased rigidity of emphysematous lungs described by Stead, Fry, and Ebert (19) and McIlroy and Christie (20) and the generally held concept derived from post-mortem findings of voluminous lungs which do not collapse and the early observations of Christie (21) that pulmonary emphysema is associated with loss of lung rigidity.

The data presented indicate that compliance varies with the amount of viscous resistance present, decreasing with increasing respiratory rate. Thus, despite the high values obtained for the static compliance of the lungs in chronic pulmonary emphysema, indicating loss of rigidity, the functional compliance was low while breathing at the resting respiratory rate. These results are, therefore, similar to those found by McIlroy and Christie (20) and are presumably due to the large element of viscous resistance which was present.

On the other hand such an explanation would not suffice for Stead, Fry, and Ebert's patients, for they were studied during static states, which should yield values comparable to those for the true compliance. Nevertheless, the low values for compliance which they reported might be due to the presence of fibrosis and congestion as in our second group of abnormal subjects, who had developed right heart failure. Here the static compliance of the lungs was much lower indicating that the lungs were stiffer and more difficult to distend, perhaps because of the development of fibrosis and vascular congestion. It is of interest that these patients had been dyspneic for the longest period of time. In those patients with apparently uncomplicated emphysema whose static compliance was within the normal range, loss of elasticity may have been obscured by the preponderance of fibrosis.

The data presented in this study suggest that there is a considerable amount of alveolar wall damage with loss of elasticity early in emphysema and that fibrosis and congestion may be present in

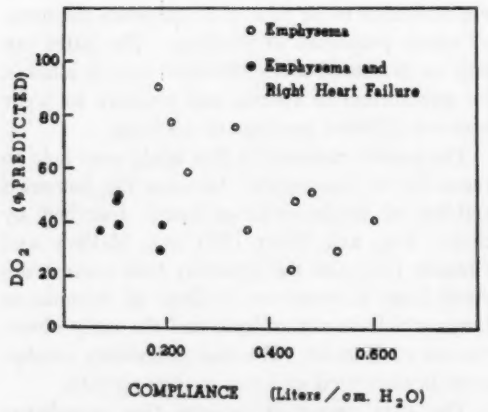

FIG. 6. THE RELATIONSHIP BETWEEN STATIC COMPLIANCE AND MAXIMAL DIFFUSING CAPACITY FOR OXYGEN, IN EMPHYSEMA AND IN EMPHYSEMA AND RIGHT HEART FAILURE

the cases with right heart failure. Since the maximal diffusing capacity has been suggested as an estimate of the amount of alveolar damage and reduction of pulmonary area available for diffusion of oxygen (*22*), it is of interest that there was a rough relationship between the static compliance and the maximal diffusing capacity for oxygen in the emphysematous patients (Figure 6). When right heart failure was present there appeared to be a disproportionate reduction in the lung compliance.

In assessing the values reported for viscous resistance, consideration must be given to the fact that the compliance is a component of the calculation, and to the observation that compliance varies with the frequency of respiration and rate of air flow in the patients who have a high element of viscous resistance. If viscous resistance is determined from a pressure-volume loop it is also apparent that the line representative of the compliance will not be a straight line joining end expiration and end inspiration, but will be a curved line proportional to the rate of air flow. Since the pressure required to overcome the viscous resistance is equal to the difference between intrathoracic pressure and the pressure required to overcome elastic resistance, this implies that the amount of viscous resistance will be over-estimated. This is particularly true for the value for turbulent resistance since this is determined at high rates of flow. On the other hand, the value for laminar resistance is obtained at lower rates of air flow and the value for the coefficient of linear resistance is probably a good approximation. Determination of pressure-flow characteristics by interruption of air flow at the mouth would be similarly affected since the static compliance measured during the period of interruption would probably be higher than the functional compliance during that particular breath. This would serve as the explanation for our observation and that reported by Fry, Ebert, and Stead (13), that a rounded slope precedes the development of the plateau in intrathoracic pressure during interruption of air flow in emphysematous patients.

Despite these considerations the high values obtained for the co-efficients of viscous resistance suggest that there is a large element of viscous resistance, both laminar and turbulent, in the subjects with pulmonary emphysema, particularly in those who had developed right heart failure. A marked increase in work was thus required in order to overcome the viscous resistance. It is of interest that there is a rough correlation between the work required to overcome viscous resistance during normal respiration and the maximum breathing capacity (Figure 7).

The beneficial effect of a single administration of nebulized bronchodilator in the patients with chronic obstructive pulmonary emphysema is em-

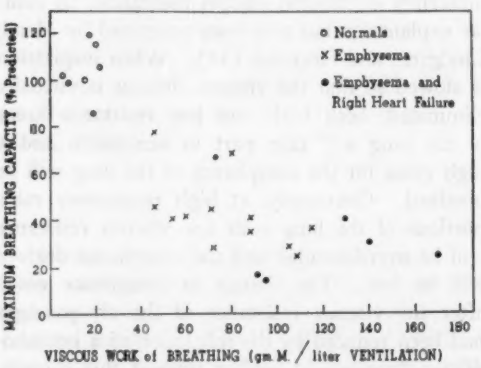

FIG. 7. THE RELATIONSHIP BETWEEN WORK REQUIRED TO OVERCOME VISCOUS RESISTANCE DURING SPONTANEOUS RESPIRATION AND THE MAXIMUM BREATHING CAPACITY IN NORMAL SUBJECTS, AND SUBJECTS WITH EMPHYSEMA, AND SUBJECTS WITH EMPHYSEMA AND RIGHT HEART FAILURE

phasized by the marked reduction of both linear and turbulent resistance to air flow that was obtained with the resulting decrease in work of breathing due to the decreased viscous resistance and increased functional compliance.

From the data reported, it is apparent that even at the resting rate of respiration, both elastic and viscous resistances are high in chronic obstructive emphysema, particularly after the development of right heart failure. An increased amount of work is therefore required in order to overcome both of these resistances. An increased rate of respiration further increases the work required to overcome both elastic and non-elastic resistance, and the increased work of breathing must therefore limit exercise tolerance and the performance of the maximum breathing capacity.

SUMMARY AND CONCLUSIONS

The physical properties of the lungs were measured in 7 normal subjects, and 18 patients with chronic obstructive emphysema, 6 of whom had developed right heart failure.

In patients with pulmonary emphysema, the compliance of the lungs varied with the respiratory rate, the functional compliance during spontaneous breathing being lower than the normal. In the normals, functional and static compliance were approximately the same. These findings in the abnormal subjects are most readily explained by the effects of a non-uniformly distributed viscous resistance. Measurements of static compliance indicated a loss of rigidity of the lungs in pulmonary emphysema and increased rigidity in cases with associated right heart failure.

Since the compliance varied with the rate of air flow in emphysema, the viscous resistance, and particularly that resulting from turbulence, may be overestimated.

At the resting rate of respiration both elastic and viscous resistances were high in emphysema, particularly, after the development of right heart failure. Any increase in respiratory rate increased the amount of work required for respiration.

A single dose of nebulized bronchodilator diminished both viscous and elastic resistance to respiration in patients with pulmonary emphysema.

ACKNOWLEDGMENTS

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THE FRACTIONATION OF CHOLESTEROL IN BODY FLUIDS BY MEANS OF SOLVENT EXTRACTION [1]

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In an earlier study (1) it was found that cholesterol absorbed from the intestinal lumen differed from cholesterol secreted into the plasma by the liver in that the former entered and circulated in plasma (for a time at least) enmeshed in a lipid aggregate (*i.e.*, as chylomicra) but the latter entered and circulated in plasma as one of its solutes. It was observed that not only could these two types of cholesterol be separated by physical methods (*e.g.*, by ultracentrifugation) but also that they were handled differently by the body. Thus cholesterol in chylomicron form was deposited in the hepatic reticuloendothelial cell but no such disposition occurred in the case of endogenously produced plasma-soluble cholesterol (2).

Indeed, it has been observed by several investigators (3–5) that treatment of sera with various fat solvents such as an alcohol-ether solution or chloroform leads to an extraction of cholesterol, the degree of which is dependent seemingly upon the increment of cholesterol which remains unbound with protein and apparently is carried in the other lipids of serum. Since a chylomicron consists of various lipids and may contain cholesterol with but little protein, it seems reasonable to believe on *a priori* grounds, that its cholesterol content might be more easily removed by suitable extracting agents than cholesterol already bound to protein. If such were the case, then analysis of appropriate extracts of a given serum might offer a means of estimating the respective amounts of cholesterol present in lipoprotein form and that present unbound to protein and ostensibly carried in a lipid aggregate.

In the present report, extraction of the intestinal lymph and of the serum of the rat under various conditions has been done. Studies of the rabbit also were made. The results suggest that any *increase* in the amount of "easily extractable" cholesterol obtained from the serum of either the normo- or hypercholesteremic rat is proportionate to an associated increase in the lipid fraction of the same serum sample.

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METHODS

I. *Cholesterol extraction of rat and rabbit sera*

The cholesterol extraction of samples of intestinal lymph and of normocholesteremic and hypercholesteremic serum was performed by one of the techniques of Macheboeuf and Sandor (3) as follows: 1 ml. of freshly obtained serum, heparinized plasma, lymph or chylomicron suspension was placed in the bottom of a heavy walled test tube of approximately 10 mm. internal diameter. One ml. of a mixture of 1 volume of absolute ethanol with 9 volumes of ether was layered over the watery fluid, the tube sealed with a paraffin-covered cork and inverted exactly 20 times. The tube was then placed upright at 4° C. for 20 hours. At the end of this time the upper ether layer was transferred to a suitable container by means of a dropper pipette. One ml. of fresh alcohol-ether (A–E) solution was carefully layered over the serum or other fluid, and immediately removed. This wash was repeated a second time. The combined extract and washings were then analyzed for cholesterol by our usual methods, as was the lower watery layer. A preliminary cholesterol and lipid analysis of each serum or lymph was obtained, the cholesterol analysis by the method of Saifer and Kammerer (6) and the lipid analysis by the method of Bragdon (7).

Fifteen normal male rats of Long Evans strain (av. wt.: 275 grams) were given 100 mg. of cholesterol dissolved in 3 ml. of olive oil by stomach tube. Blood samples obtained by bleeding ether-anesthetized animals from the tail before and 3, 6 and 12 hours after the feeding were analyzed for cholesterol and then submitted to the extraction procedure. Three unfed control rats were bled at the same time intervals and the samples were similarly treated.

Six normal rabbits (av. wt.: 1609 grams) were given 400 mg. of cholesterol dissolved in 12 ml. of olive oil by stomach tube. Blood samples were obtained before and at 3, 6, 12 and 24 hours after the feeding. A portion was reserved for cholesterol analysis and the remainder submitted to the extraction procedure.

II. *Cholesterol extraction of rat intestinal lymph*

Three series of normal male rats (Long Evans strain) were used in this experiment. Series I (6 rats) were given 3 ml. of H_2O by stomach tube; Series II (19 rats) were given 3 ml. of olive oil by stomach tube; Series III (26 rats) were given 100 mg. of cholesterol dissolved in 3 ml. of olive oil. The intestinal lymph of the rats of all three series was collected for 24 hours by previously described methods (1) and a sample of each lymph was analyzed for cholesterol and then submitted to the extraction procedure.

III. *Cholesterol extraction of rat hypercholesteremic sera*

A. *Triton induced hypercholesteremia (rat):* Ten male rats of Long Evans strain (av. wt.: 291 grams) were injected intravenously with 100 mg. of Triton WR-1339 after a fasting period of 20 hours and blood samples obtained before and 6 and 24 hours after injection were analyzed for cholesterol and lipid and then submitted to the extraction procedure.

B. *Cholate induced hypercholesteremia (rat):* Twelve male rats of Long Evans strain (av. wt.: 280 grams) were submitted to biliary obstruction which induced hypercholesteremia by elevation of the plasma cholate concentration (8). Blood samples obtained before and 6 and 24 hours after biliary obstruction were analyzed for cholesterol and lipid and then submitted to the extraction procedure.

RESULTS

A. *The cholesterol extraction of rat and rabbit sera before and after oral ingestion of cholesterol*

When the sera of 15 normal rats which had been fasted for 18 hours was submitted to the extraction procedure with A-E solution, little of its cholesterol was removed. As Table I demonstrates, the average cholesterol concentration of the A-E solution was only 1.4 mg. per 100 ml. or about 3 per cent of the total plasma cholesterol concentration (61 mg. per 100 ml.). However, when the sera of the same rats were again submitted to extraction three hours after the oral ingestion of 100 mg. of cholesterol in 3 ml. of olive oil, the amount of extracted cholesterol more than tripled (see Table I) and equalled 8 per cent of the initial total plasma cholesterol concentration. These sera also exhibited turbidity and chylomicra. Although extraction of similarly turbid sera obtained six hours after feeding gave a value almost the same (5.8 mg. per 100 ml.) as that obtained after three hours, a considerably greater fraction of

TABLE I

The cholesterol extraction of serum of the cholesterol fed rat and rabbit

	Cholesterol (*mg./100 ml.*)					
	Rats (15) Av. wt. = 303 grams			Rabbits (6) Av. wt. = 1,609 grams		
	Av.	Range	S.E. mean	Av.	Range	S.E. mean
Before feeding						
Serum	61	49–91		94	66–190	
A-E Sol.	1.4	0.3–2.8	±0.08	5.5	4.1–6.3	±0.3
A-E/S*	0.02	0.01–0.04	±0.01	0.07	0.03–0.09	±0.01
3 Hours after feeding						
Serum	60	45–68		71	60–83	
A-E Sol.	4.82	3.3–9.6	±0.65	5.9	4.6–6.8	±0.3
A-E/S	0.08	0.06–0.15	±0.01	0.05	0.07–0.10	±0.01
6 Hours after feeding						
Serum	71	59–88		82	66–98	
A-E Sol.	5.8	1.8–23.4	±1.68	5.8	3.2–7.7	±0.6
A-E/S	0.08	0.02–0.31	±0.01	0.07	0.03–0.09	±0.01
12 Hours after feeding						
Serum	74	49–91		79	59–95	
A-E Sol.	12.2	5.5–27.0	±5.7	7.3	4.8–14.0	±1.1
A-E/S	0.17	0.07–0.36	±0.01	0.09	0.06–0.16	±0.01
24 Hours after feeding						
Serum				133	81–165	
A-E Sol.				11.4	3.8–28.4	±3.5
A-E/S				0.08	0.03–0.18	±0.01

* A-E/S: Ratio of cholesterol in solvent/cholesterol in original serum.

TABLE II

The cholesterol extraction of the intestinal lymph of the rat

No. of rats	Av. wt. (*gm.*)		Lymph (24-Hour collection) Volume (*ml.*)	Cholesterol (*mg./100 ml.*) Lymph	A-E Sol.	A-E/L*
			(Rats given 3.0 ml. of H_2O)			
6	294		25.0	43.0	6.4	0.14
		Range	(20.0–29.0)	(26.0–57.0)	(1.0–11.2)	(0.04–0.21)
		S.E. mean		±4.3	±1.6	±0.03
			(Rats given 3.0 ml. of olive oil)			
18	302		37.0	37.0	10.2	0.263
		Range	(18.0–59.0)	(17.4–54.0)	(0.8–23.4)	(0.04–0.65)
		S.E. mean		±1.1	±1.7	±0.04
			(Rats given 3.0 ml. of olive oil + 100 mg. of cholesterol)			
25	316		37.0	92.0	41.7	0.403
		Range	(15.0–83.0)	(44.0–202.0)	(4.5–124.0)	(0.10–0.83)
		S.E. mean		±6.1	±6.3	±0.04

* A-E/L: Ratio of cholesterol in solvent/cholesterol in original lymph.

cholesterol (16.5 per cent) was extracted from the turbid, chylomicronemic sera obtained from the rats 12 hours after the ingestion of the cholesterol. As Table IV shows, the amount of cholesterol capable of being extracted from the sera of unfed control rats does not change after repeated bleedings, and these sera also never exhibited turbidity or chylomicronemia. These results indicate that although the total cholesterol concentration of the sera of rats does not change markedly after cholesterol ingestion, the physicochemical status of the cholesterol is markedly altered. This is shown by the extraction of a portion of the serum cholesterol at 12 hours following the intake of cholesterol almost nine times as great as may be extracted by alcohol-ether extraction before feeding. This alteration in extraction appeared to proceed *pari passu* with the degree of lipemia, as judged by visual estimation of the turbidity of serum samples.

The results obtained from the extraction of sera of cholesterol fed rabbits were somewhat similar except the increase in extraction was delayed. Thus (see Table I), no significant increase occurred either in the total cholesterol concentration or in the extractable portion either at 3, 6 or 12 hours after the oral ingestion of the cholesterol. Similarly, no discernible increase in turbidity was observed nor were chylomicra observed. At the end of 24 hours, the sera were moderately turbid and chylomicronemic and contained considerably more cholesterol (133 mg. per 100 ml.). The amount of cholesterol extracted from these sera was about double that able to be extracted prior to cholesterol feeding. The percentage of extraction, however, did not increase appreciably because of the actual increase in cholesterol concentration of the sera.

B. *The cholesterol extraction of intestinal lymph of the rat*

Unchanged intestinal lymph. The intestinal lymph of the six rats given only H_2O contained considerable cholesterol (43 mg. per 100 ml.), and a moderate amount of it could be extracted with the A-E solution, the latter's concentration of cholesterol being 6.4 mg. per 100 ml. or approximately 14 per cent of the initial lymph concentration (see Table II). The lymph itself was moderately turbid and exhibited a number of chylomicra.

The lymph samples of the 18 rats given 3 ml. of olive oil were very turbid and contained myriads of chylomicra. The average cholesterol concentration of these lymphs was 37 mg. per 100 ml. (see Table II) and a considerable portion was capable of extraction. Thus the A-E solution (see Table II) contained 10.2 mg. per 100 ml. or about 26 per cent of the initial lymph cholesterol concentration.

The lymph samples of the 25 rats given 100 mg. of cholesterol in 3 ml. of olive oil were similar to

the above samples in the intensity of their turbidity and chylomicron content. The average cholesterol content of this lymph was 92 mg. per 100 ml. and compared to the preceding lymphs, a greater portion of its cholesterol was capable of extraction. The A-E solution (see Table II) contained 41.7 mg. per 100 ml. or approximately 40 per cent of the initial lymph concentration.

Ultracentrifuged portions of intestinal lymph. The five lymph samples which were collected from rats given 100 mg. of cholesterol in 3 ml. of olive oil separated on ultracentrifugation into two distinct phases. The upper phase which consisted of a dense white cake having a volume of less than 1.0 ml. was removed and suspended by stirring in 5 ml. of H_2O. The lower phase was only slightly turbid and contained only a few chylomicra. It resembled the intact lymph obtained from the water-fed rats.

The cholesterol concentration of the A-E solution after the latter's contact with the original intact lymph averaged 46 mg. per 100 ml. (see Table III) or about 38 per cent of the initial lymph concentration.

When the top phase of the ultracentrifuged lymph samples was redissolved and then exposed to the A-E solution, the latter obtained an average cholesterol concentration of 55 mg. per 100 ml. or 64 per cent of the cholesterol concentration of the initial concentration of the redissolved top phase. On the other hand, similar exposure of the lower phase of the ultracentrifuged lymph to A-E solution resulted in an average cholesterol content of only 8.0 mg. per 100 ml. in the latter solution or about 15 per cent of the initial concentration in the lower phase. Clearly then, the cholesterol content of the chylomicra was far more easily extracted than the cholesterol predominantly in solution in the lower phase of the ultracentrifuged lymph.

C. *The cholesterol extraction of rat hypercholesteremic sera*

Two entirely different types of hypercholesteremic serum were studied. The first hypercholesteremic serum was obtained from rats subjected to bile obstruction for 24 hours. This serum was perfectly clear, exhibited no chylomicra but contained about 2 to 3 times more total lipid than the serum of the normal fasted rat. Its lipoproteins, however, exhibited a relatively low flotation rate (9). The second type of hypercholesteremic serum, obtained from rats injected with Triton six and 24 hours previously, was intensely turbid and contained far more total lipid (approximately twice as much as the serum from rats with ligated bile ducts). The turbidity was due to particles which, although not visible even at 1000 diameters magnification, could be separated in great part by centrifugation at 20,000 r.p.m. (r.c.f. 25,000 to 40,000). Such particles ostensibly are rich in lipids other than cholesterol which probably accounts for the marked alteration in the lipoprotein spectrum occurring after Triton administration (10).

As Table IV indicates, layering with A-E solution resulted in significantly increased extraction of cholesterol from the sera of rats obtained six hours after biliary obstruction and also resulted in a considerable increase in extraction from the

TABLE III

The cholesterol extraction of intestinal lymph before and after ultracentrifugation

		Intestinal lymph collection (*24 hours*)									
						Ultracentrifuged lymph					
						Cholesterol (*mg./100 ml.*)					
		Unchanged lymph				Upper layer*			Lower layer		
			Cholesterol (*mg./100 ml.*)								
Rat	Weight (*gm.*)	Vol. (*ml.*)	Lymph	A-E Sol.	A-E/L	Intact layer	A-E	A-E/I.L.	Intact layer	A-E	A-E/I.L.
18	290	28	120	44	0.37	73	49	0.67	38	5.5	0.15
19	280	26	94	30	0.32	61	41	0.67	37	7.5	0.22
21	254	16	161	55	0.34	125	79	0.63	98	14.0	0.14
22	276	26	112	55	0.49	85	51	0.60	31	5.0	0.16
Av.	275	24	122	46	0.38	86	55	0.64	51	8.0	0.17

* Top layer obtained from ultracentrifugation was resuspended in 5.0 ml. of physiological saline solution.

TABLE IV

The cholesterol extraction of serum of hypercholesteremic rats

	Control rats (6)			Bile obstructed rats (11)			Rats injected with Triton (10)		
	Av.	Range	S.E. mean	Av.	Range	S.E. mean	Av.	Range	S.E. mean
Before experiment									
Serum	52	44–56		56	50–64		57	49–71	
A-E Sol.	1.1	0.5–1.8	±0.3	0.98	0.6–1.4	±0.08	1.7	0.6–2.7	±0.26
A-E/S	0.02	0.01–0.03	±0.01	0.02	0.01–0.03	±0.005	0.03	0.01–0.04	±0.01
Six hrs. after experiment begun									
Serum	48	42–50		63	54–70		169	146–207	
A-E Sol.	1.6	1.5–1.7	±0.03	2.5	0.8–4.4	±0.04	63.0	28–92	±6.1
A-E/S	0.04	0.03–0.04	±0.02	0.04	0.02–0.07	±0.06	0.37	0.18–0.48	±0.03
24 hrs. after experiment begun									
Serum	55	52–58		131	95–171		341	299–422	
A-E Sol.	1.3	1.2–1.5	±0.07	12.1	2.8–32.0	±2.7	145	112–174	±5.3
A-E/S	0.023	0.02–0.03	±0.03	0.10	0.03–0.29	±0.02	0.43	0.31–0.52	±0.03

sera obtained after 24 hours of obstruction. Thus the average cholesterol content extracted from the 24-hour sera was 12.1 mg. per 100 ml. and the extraction ratio rose from 2 to 10 per cent.

The sera obtained from Triton-injected rats contained far more extractable cholesterol both at six and 24 hours after injection. The A-E solution contained 63 mg. at six and 145 mg. of cholesterol per 100 ml. at 24 hours, respectively, after injection of Triton (Table IV). Undoubtedly this greater degree of extraction compared with that of the previous type of hypercholesteremic serum was due in part to the greater content of cholesterol in the serum of the Triton-injected rats. However, the extraction ratio similarly increased markedly being 37 and 43 per cent at six and 24 hours, respectively.

DISCUSSION

In the present study, the cholesterol of intestinal lymph of the unfed rat was found to be considerably more extractable than its counterpart in serum, possibly due in part to the fact that some of the cholesterol in lymph (perhaps that portion reabsorbed from that previously excreted into the intestine) (11) travels in a lipid particle (1). Also, there is considerably less protein available in intestinal lymph for lipoprotein bondage than is the case with serum (12). After fat or cholesterol feeding, the amount of cholesterol extractable from intestinal lymph increased markedly and this *net increment* was observed to be present in the chylomicra (*i.e.*, in the upper layer of the ultracentrifuged lymph). The extraction of the lower layer of the ultracentrifuged lymph of the cholesterol-fat-fed rat however (see and compare Tables II and III) yielded about the same ratio of extracted to total cholesterol as that of the intact lymph of the fasted rat. In other words, cholesterol enmeshed in lipid appeared to be more readily extractable than cholesterol attached to protein as a serum soluble lipoprotein.

The rise observed in the cholesterol extraction ratio of hypercholesteremic and hyperlipemic sera is a phenomenon which confirms the observations of Forbes, Dillard, Porter, and Petterson (4). They noted that a greater proportion of the cholesterol of sera of nephrotic individuals was extracted by chloroform treatment than was extracted from the cholesterol present in sera of normal individuals. In both of the experimentally induced hypercholesteremic states of this study and also in the nephrotic state (4), an excess of lipid is also present in the serum. The conclusion then seems warranted that the cholesterol present in a hyperlipemic serum is more easily extractable than the cholesterol present in a normolipemic serum.

Although the present experiments suggest that cholesterol can be extracted to some extent even when not existing in visible chylomicron form, as was found when the chylomicron free serum of rats with biliary obstruction was treated with the A-E solvent, nevertheless, the cholesterol of those

lymph and serum samples exhibiting the greatest concentration of lipid aggregates was found to be most readily extracted. Since the presence of lipid aggregates necessarily implies a state of supersaturation of the plasma with regard to at least one of the lipid components, it is possible that two causes may contribute independently to the extractability of cholesterol (13). In other words, the increased amount of total lipid may 1) remove some of the cholesterol from soluble lipoprotein form into aggregate form, and 2) alter some of the remaining soluble lipoprotein-cholesterol so that it is more readily extractable.

In the absence, however, of an endogenous form of hypercholesteremia or hyperlipemia, changes in the per cent of serum cholesterol which may be extracted might only be expected following changes in the lipid concentration of plasma. The present experiments involving both the rat and rabbit fed cholesterol and fat suggest that such changes in cholesterol extractability may occur during the post alimentary period.

SUMMARY

Extraction with an alcohol-ether solution of various types of sera and lymph samples obtained from rats permitted a partial extraction of their cholesterol content. The degree of extractability of cholesterol of any sample appeared to be a function dependent in part at least upon the increase in serum lipid.

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FAMILIAL HYPOGLYCEMIA PRECIPITATED BY AMINO ACIDS

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Mann and Magath (1) in 1922 first described the clinical symptoms associated with hypoglycemia, and in 1924 Harris (2) reviewed its relationship to hyperinsulinism. Since then, numerous articles have appeared in the literature reviewing the causes of hypoglycemia, (3–6) describing individual cases and suggesting various surgical and medical methods of management.

Although spontaneous hypoglycemia is evidence of abnormal carbohydrate metabolism, this abnormality may be due to a variety of diseases.

The present communication describes three cases of hypoglycemia occurring in one family, and one unrelated case, in which convulsions and profound lowering of the blood glucose were induced by the administration of proteins or amino acids.

We believe this abnormal relationship between amino acids and glucose metabolism has not been previously described, and will be of great interest, not only to the clinician, but also to the biochemist and physiologist investigating carbohydrate and protein metabolism.

CASES

Case 1. A female infant was admitted to The Hospital for Sick Children, Great Ormond Street on 29th September 1954, at the age of 4 months for investigation of convulsions. Her mother's pregnancy and labor were normal. Her birth weight was 7 lbs. The infant was breast fed for 10 weeks and then given half cream cow's milk feeds with added sugar. At 3 months she began to have generalized convulsions. She had two or three fits every day and these usually occurred about midway between feeds but not after waking in the mornings. The fits lasted from a few minutes to half-an-hour.

Examination showed a plump 12 lb. 3 oz. infant with no abnormal physical signs. Her mental development was normal for her age. Since her sister (case 2) had convulsions associated with hypoglycemia, fasting blood was taken and this showed blood sugar levels varying from 40 to 60 mg. per 100 ml. The levels of blood sugar before feeds showed a gradual decline throughout the day—the fasting level being around 50 mg. per 100 ml. and by 10 p.m. the level had dropped to around 30 mg. per 100 ml.

An electro-encephalogram, carried out when the blood sugar level was 38 mg. per 100 ml., was normal.

The plasma level of insulin-like activity was estimated by Dr. P. J. Randle (7, *cf.* 8, 9) and was found to be normal.

A high protein low carbohydrate diet (cow's milk with added casein) was then given and she immediately began to have convulsions which occurred at about one hour after each feed. On "Soylac," a soya flour synthetic milk, she had numerous convulsions, her fasting blood sugar was only 20 mg. per 100 ml. and this tended to drop slightly during the day. She was then fed with expressed breast milk, the levels of blood sugar before feeds were higher and the baby was free from fits for the first time. Cow's milk diluted with three parts of water and with added sugar had the same effect. Mixed feeding was started, care being taken to see that no large amount of protein was given without carbohydrate being given at the same time. She has remained well, having about one fit every six weeks. Her mental and physical development are normal.

Case 2. A female infant, elder sister of case 1, was admitted to The Hospital for Sick Children, Great Ormond Street on 30th May 1953 at the age of 2½ months. Her birth weight was 7 lb. 1 oz. and her mother's pregnancy and labor were normal. She was breast fed for two months and then given full cream dried cow's milk feeds. After one week she began having generalized convulsions dating from the time when extra sugar was omitted from her feeds. During the fits she became flushed, sweated, and lost consciousness. She had about twelve fits in the three weeks prior to admission. No history of their relation to feeds could be obtained. Examination showed a small infant who weighed 7 lb. 10 oz. There were no abnormal signs and her mental development was within the normal range for her age. The C.S.F. was normal apart from a sugar value of 25 mg. per 100 ml. An E.E.G. was normal. The fasting blood sugar levels varied from 32 mg. per 100 ml. to 50 mg. per 100 ml. Blood sugar levels just before meals were in the range of 40 to 65 mg. per 100 ml.

A laparotomy was performed; the pancreas was normal in size but rather firm and portions of the tail of the pancreas and of the liver were removed for histological study. There was no evidence of any neoplastic or hyperplastic change in the islets and no significant pathological change in the islet cells except that there was some enlargement, vacuolation and loss of granular dis-

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tinction in the β-cells which was interpreted as due to edema, a change which was also noted in the interstitial tissue. Both α- and β-cells were present in normal numbers. The histology of the liver was normal and there was no evidence of glycogen storage disease.

She was sent home in October 1953 on a normal diet with only an 8-hour fast at night, as during the latter part of her stay in hospital there was a spontaneous improvement in her condition. Two months later she was admitted with intestinal obstruction due to herniation of small intestine through the mesocolon. One foot of small bowel was resected, and although her condition at the time was poor, recovery was uneventful. After recovery she was put on normal mixed feeding and had no fits. During the following year she had a fit about once every 3 months. She sat up at ten months, walked at one year and began to talk at 20 months.

She was re-admitted for further investigation on April 12th 1955.

Case 3—is the father of cases 1 and 2 and is 27 years old. He had fits as a baby. From 10 months of age until he was 13 years old he would occasionally fall into a deep sleep which lasted from a few moments to 3 or 4 days; this was considered to be a form of narcolepsy but the hospital records could not be traced. He has remained well since then and has no sweating or sensations which could be attributed to hypoglycemia. His fasting blood sugar was estimated on three occasions and found to be 64, 46 and 48.5 mg. per 100 ml., respectively (the first two values are of "apparent sugar" estimated by the method of Folin and Wu, the third is "true sugar" estimated as described below). His plasma level of insulin-like activity was normal (7, *cf.* 8 and 9).

Family history: Cases 1 and 2 are the only children of case 3. There were no still-births or miscarriages. Repeated attempts to trace other members of the father's family have failed. There is no history of epilepsy in the mother's family.

Case 4—was the only child of healthy Cypriot parents. One of the mother's uncles is said to have had fits but no further details could be obtained. He was admitted to The Hospital for Sick Children, Great Ormond Street on 26th March 1952 at the age of 5 months for the investigation of convulsions. His mother's pregnancy and labor were normal. His birth weight was 6 lb. 14 ozs. He was breast fed until he was 3½ months old when he was given full cream dried cow's milk feeds with added sugar. When he was 2 months old he began to have fits. At first he went pale without sweating, rolled up his eyes and did not respond to external stimuli. These fits occurred 2 to 3 times daily, usually during a feed, and they lasted about two minutes. Over a period of a few weeks he began to have more frequent and generalized convulsions unrelated to feeds. He was then admitted for further investigation.

Examination showed a large hypotonic infant weighing 12 lb. 2 oz. He did not hold up his head neither did he attempt to grasp an object, nor follow a light. There were no other abnormal signs.

The cerebro-spinal fluid was normal apart from a sugar value of 20 mg. per 100 ml. His fasting blood sugar levels ranged from 17 to 50 mg. per 100 ml. Preprandial blood sugars remained between 35 and 45 mg. per 100 ml.

On 6th January 1953 a laparotomy was carried out. The pancreas appeared large but otherwise normal. The distal two-thirds were removed. There was about four times the normal amount of islet tissue present, this was partly grouped in well formed islets but in addition there were islet cells grouped in strands and small acini infiltrating between the acini of exocrine tissue. There was no evidence of neoplastic change and no increase in interstitial tissue. The majority of cells were β-cells and a minority were α-cells.

A liver biopsy carried out at the same time showed no evidence of glycogen storage disease. The plasma level of insulin-like activity was six times normal before operation, twenty months after operation it was five times normal (7, *cf.* 8 and 9). Following operation his fasting blood sugar fell over 3 months from 50 to 25–30 mg. per 100 ml. He was given cortisone for 8 months and during this time had no fits. Since then he has had only one fit every four to six weeks apart from a period of 2 to 3 months when he had 2 to 3 fits a week. He is given frequent meals and phenobarbitone.

Although his development appeared to be normal until the onset of fits at two months of age, he is now three years and four months old and is grossly retarded. He began to walk with help when two years old, but is still unable to walk unaided. He does not speak, has to be fed and has no control over micturition and defecation.

MATERIALS AND METHODS

The blood sugar concentration was followed in cases 1, 2, 3, and 4 and in normal subjects after feeding glucose, casein, gelatine, leucine, tyrosine, or isovaleric acid and after injecting insulin, glucagon or adrenaline.

L-leucine puriss and L-tyrosine were used as purchased from L. Light & Co. Ltd. They were suspended in a little water or in 3 per cent methylcellulose which was flavored with saccharin and oil of orange and frozen to an "ice cream."

Isovaleric acid, laboratory reagent grade, was dissolved in a slight excess of aqueous sodium bicarbonate solution and chilled.

Casein ("Casilan," Glaxo) and gelatine were suspended in a little water.

Biodeminrolit G (Permutit, Ltd.) was saturated with carbon dioxide and kept in a tightly closed jar.

Sodium tungstate: a 10 per cent aqueous solution was used.

Sulphuric acid: 0.66 N.

Alkaline copper reagent (10):

A: $CuSO_4 \cdot 5H_2O$: 2 g., Na_2SO_4: 18 g., water to 100 ml.

B: Na_2CO_3: 12 g., Rochelle salt 6 g., $NaHCO_3$: 8 g., Na_2SO_4: 72 g., water to 400 ml.

These were mixed in the ratio of one part A to four parts B immediately before use.

Arsenomolybdic acid was prepared by the method of Nelson (11).

Blood sugar estimation. After an overnight fast (7 to 14 hours) a specimen of capillary blood was collected by finger-prick or heel-prick into a bottle containing fluoride and oxalate. The test-substance was fed, the time taken to swallow it was noted, and blood samples were taken 10, 20, 30, 45, 60, and 90 minutes after all the substance had been ingested.

The blood sugar estimations were carried out as soon as all the blood samples had been collected. Four-tenths ml. of each sample (or less if the blood sample was unusually small) was added from a pipette calibrated "to contain" to 6.8 ml. of water in a centrifuge tube and mixed. Four-tenths ml. of sodium tungstate and 0.4 ml. of sulphuric acid were added to each tube and the contents well shaken. After centrifuging, the supernatant from each tube was shaken with Biodeminrolit G (about 0.75 ml. in volume) for 5 minutes. The sugar in the supernatant was estimated by the alkaline copper arsenomolybdate method and compared with a standard solution of glucose treated similarly (10, 11). The color intensities were read against the blank in a photo-electric colorimeter using an Ilford 604 filter ($\lambda_{max} = 520$ mμ).

Blood amino-acid values. These were determined by the method of West, Wilson, and Eyles (12).

Paper chromatography. For investigating the blood sugars the tungstic acid filtrate, after treatment with Biodeminrolit G, was examined by paper chromatography (13, 14). Urine was treated with Biodeminrolit G and similarly examined.

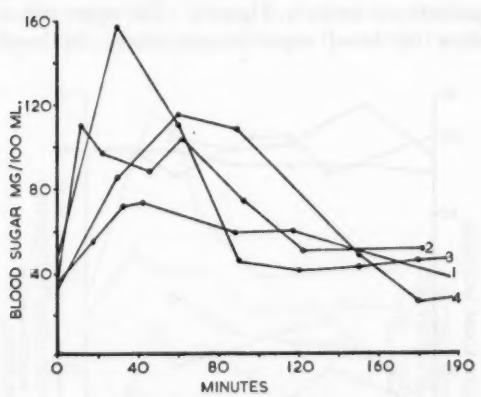


FIG. 1. GLUCOSE TOLERANCE CURVES

Curve 1: true blood sugar of case 1 fed 14 g. glucose (1.6 g. per kg. body weight) over 13 mins. Curve 2: true blood sugar of case 2 fed 15.5 g. glucose (1.45 g. per kg.) over 2 mins. Curve 3: apparent blood sugar of case 3 fed 50 g. glucose (0.8 g. per kg.). Curve 4: apparent blood sugar of case 4 fed 16 g. glucose (1.4 g. per kg.). The blood sugar did not drop significantly below the fasting level during the 5 hours following ingestion of glucose.

For amino acid investigation venous blood was collected and the serum passed through a column of Zeo Karb 225 (a sulphonated polystyrene resin). Proteins, sugar, acids and all other non-basic components were washed away with water, and the amino acids desorbed with 5M-ammonia solution. The ammonia eluate was evaporated in vacuo and the residue examined by two-dimensional paper chromatography without further treatment. Urinary amino acids were examined by two-dimensional paper chromatography (13) after electrolytic desalting.

RESULTS AND INTERPRETATION

Glucose tolerance

The glucose tolerance curves for the four subjects are shown in Figure 1. The values for cases 1 and 2 represent "true blood sugar" (see below) and were determined by the method given above, those for cases 3 and 4 represent "apparent blood sugar" determined by a modification of the method of Folin and Wu (15, 16). All four show a normal rise and fall, the blood sugar returning to the fasting level within 2½ hours with no evidence of an abnormal fall within 5 hours.

Effect of hormones

Glucose insulin tolerance test (17). Case 2: Glucose (10 g.) was given by mouth and, simultaneously, 1.0 unit of insulin intravenously. The fasting blood sugar was 28.5 mg. per 100 ml. and rose to 91 mg. per 100 ml. at 30 minutes falling to 38 mg. per 100 ml. at 90 minutes.

Case 4: Glucose (19 g.) was given by mouth and 1.1 units of insulin intravenously. The fasting blood sugar was 31 mg. per 100 ml. and rose to 121 mg. per 100 ml., at 30 minutes, falling to 34 mg. at 60 minutes.

Adrenaline tolerance test. Adrenaline (0.2 mg.) was given subcutaneously after an 8-hour fast to cases 1, 2, and 4. The blood sugar level rose in the first half-hour from 27 to 45 mg. per 100 ml. in case 1, from 45 to 140 in case 2 and from 52 to 115 in case 4. The blood sugar was still high after 2 hours (50, 93, and 87 mg. per 100 ml., respectively).

Glucagon. After an 8-hour fast glucagon [2] was given intramuscularly to cases 1 and 4 (7,500 and 10,000 cat units, respectively). In case 1 the blood sugar level rose from 30 to a peak of 94 mg.

[2] This was supplied by Eli Lilly & Co., to whom our thanks are due.

per 100 ml. in 15 minutes, followed by a steady fall to 38 mg. per 100 ml. at 90 minutes. In case 4 the blood sugar rose from 31 to 92 mg. per 100 ml. in 30 minutes and fell to 35 mg. per 100 ml. by 2 hours. Case 1 was given 14 gm. of casein over 30 minutes and immediately after this had been ingested 7,500 cat units of glucagon were given by intramuscular injection. The fasting blood sugar was 32 mg. per 100 ml. and after the casein had been given it fell to 19 mg. per 100 ml. Half-an-hour after the injection of glucagon the blood sugar level had risen to 76.5 mg. per 100 ml. and 2 hours later still it was 90.5 mg. per 100 ml.

A.C.T.H. and cortisone. A.C.T.H. gel (10 mg.) was given intramuscularly twice daily for 10 days to case 1. The blood sugar level was higher in the mornings but fell to its previous level later in the day. Although there were no convulsions during this period A.C.T.H. did not cause any striking alteration of blood sugar levels throughout the day. Three weeks later 10 mg. of cortisone was given 5 times daily for 3 days. No effect on the blood sugar levels was noted.

A.C.T.H. (10 mg.) was given by intramuscular injection 6 hourly to case 2 for 6 days and later 100 mg. cortisone was given daily by mouth in divided doses for 11 days. In both instances there was a slight rise in blood sugar levels throughout the day and the patient had no fits.

Cortisone (20 mg.) was given twice daily for 18 days to case 4 and then the dose was increased to 60 mg. daily. After this the fasting blood sugar rose to 40 to 61 mg. per 100 ml. and the levels of blood sugar before feeds varied between 75 and 107 mg. per 100 ml. Before cortisone was given the fasting blood sugar varied from 17 to 50 mg. per 100 ml. and the blood sugar levels ranged from 35 to 45 mg. per 100 ml. throughout the day. The cortisone was given for 8 months and during this time he had no fits.

In cases 1, 2, and 4 there was no evidence of hyper-sensitivity to insulin and the hyperglycemic response to adrenaline and glucagon was normal. Casein did not prevent the hyperglycemic effect of glucagon in case 1.

A.C.T.H. and cortisone were given in too small doses and for too short a period to case 1 for any conclusions to be drawn. In case 2 although there was no striking alteration in blood sugar levels clinical improvement was evident. Cortisone effected an improvement both in the clinical state and in the levels of blood sugar in case 4.

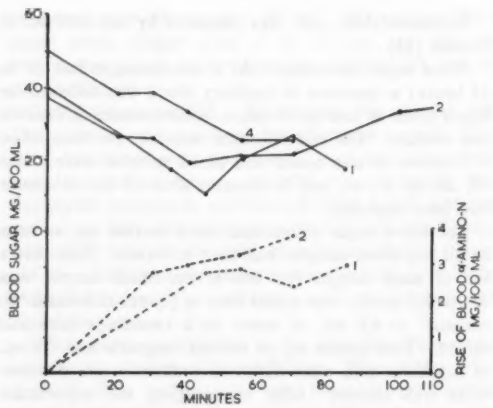


FIG. 2. BLOOD SUGAR (——) AND AMINO-NITROGEN RISE (– – –) OF CASES 1, 2 AND 4 AFTER BEING FED CASEIN

Case 1 fed 14 g. casein (1.5 g. per kg. body weight) over 25 mins. Case 2 fed 16 g. casein (1.5 g. per kg.) over 10 mins. Case 4 fed 24 g. casein (1.5 g. per kg.) over 10 mins.

Effect of protein

The effects of feeding casein to three fasting patients are shown in Figure 2. The upper curves show the blood sugar concentrations, the lower

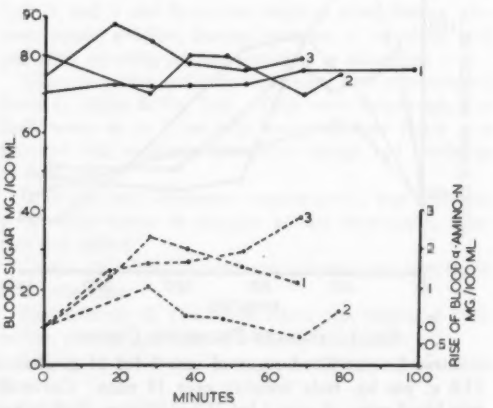


FIG. 3. BLOOD SUGAR (——) AND AMINO-NITROGEN RISE (– – –) OF THREE NORMAL SUBJECTS AFTER BEING FED CASEIN

1—was fed 16 g. (1.5 g. per kg. body weight) over 8 mins., 2—was fed 14 g. (1.5 g. per kg.) over 8 mins., 3—was fed 26 g. (1.5 g. per kg.) over 18 mins.

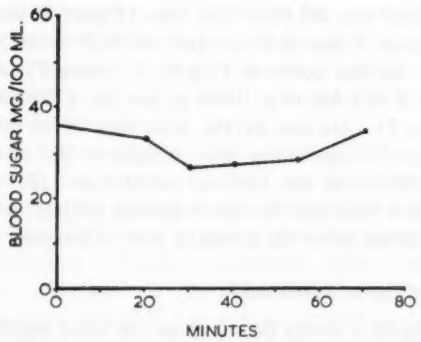


FIG. 4. BLOOD SUGAR OF A FIFTH CASE OF HYPOGLYCEMIA AFTER BEING FED 17.6 g. OF CASEIN (1.5 g. PER KG. BODY WEIGHT) OVER 10 MINS.

This child's condition had been diagnosed as spontaneous idiopathic hypoglycemia.

curves the changes in the corresponding blood amino acid concentrations. Figure 3 shows the blood sugar concentrations and the changes in blood amino acid concentration when casein was fed to fasting normal subjects. The method of measuring blood amino acid levels is adequate for changes in level after feeding protein, but cannot measure absolute amino acid concentrations with any accuracy (12).

The casein had a marked hypoglycemic effect on the three patients, but none on the normal subjects, yet the rise in blood amino acids was similar in both groups. The blood amino acid peak coincided in time with the minimum of the blood sugar concentration.

The effect on the blood sugar of feeding a normal subject a test dose of glucose alone and, next day, an equal dose of glucose with casein added is shown in Figure 5, together with the change of blood amino acid level. In case 1 a test dose of 13 g. casein and 14 g. glucose caused a slight initial rise in the blood sugar level from 47 to 58 mg. per 100 ml. after 10 minutes, followed by a profound fall to 26 mg. per 100 ml. at 20 minutes and 18 mg. per 100 ml. at 45 minutes. The blood sugar rose to 31 mg. per 100 ml. after 60 minutes. The results of the casein-glucose tests were, however, more variable than the other tests.

Gelatine had only a slight hypoglycemic effect on case 1, and tyrosine none at all (Figure 6). The rise in blood amino acids was as rapid after feeding gelatine as after casein.

Effect of leucine

Leucine caused a profound fall in blood sugar in the four patients, but had no comparable effect in normal subjects (Figure 7). Generally the blood sugar concentration in the patients dropped to about half the fasting value 30 to 45 minutes after all the test dose had been ingested, and

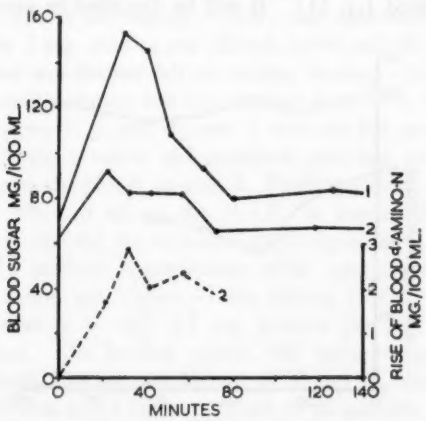


FIG. 5. 1: BLOOD SUGAR OF A NORMAL SUBJECT AFTER BEING FED 12 g. GLUCOSE ALONE (1.65 g. PER KG. BODY WEIGHT) OVER 20 MINS. 2: (——) BLOOD SUGAR AND (---) BLOOD AMINO-NITROGEN RISE OF THE SAME SUBJECT AFTER BEING FED 12 g. GLUCOSE AND 11 g. CASEIN OVER 12 MINS.

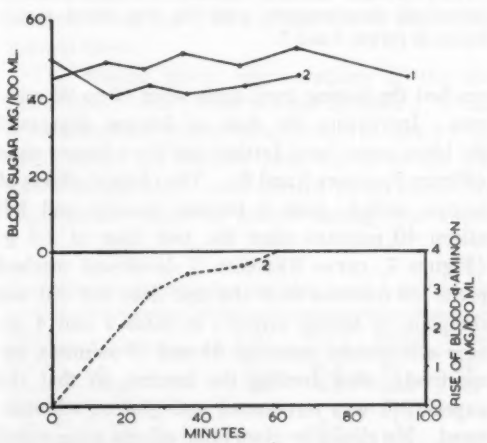


FIG. 6. 1: BLOOD SUGAR OF CASE 2 AFTER BEING FED 1 g. TYROSINE OVER 5 MINS. 2: BLOOD SUGAR (——) AND BLOOD AMINO-NITROGEN RISE (---) OF CASE 2 AFTER BEING FED 16 g. OF GELATINE (1.5 g. PER KG.) OVER 6 MINS.

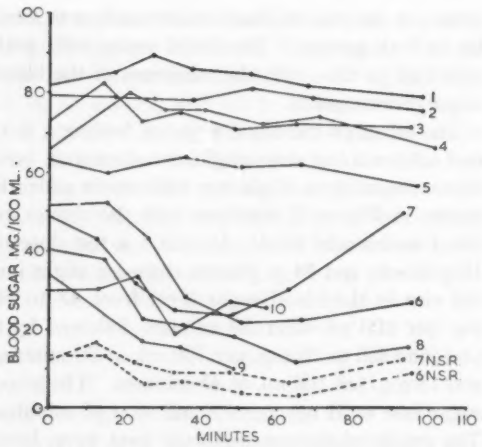


FIG. 7. BLOOD SUGAR (——) OF THE FOUR CASES OF HYPOGLYCEMIA (CURVES 6, 7, 8, 9, 10) AND OF FIVE NORMAL INDIVIDUALS (CURVES 1, 2, 3, 4, 5) AFTER BEING FED L-LEUCINE

1: Fed 10 g. leucine (.15 g. per kg. body weight) over 7 mins.
2: Fed 12.7 g. leucine (.15 g. per kg.) over 7 mins.
3: Fed 1.4 g. leucine (.125 g. per kg.) over 6 mins.
4: Fed 2.1 g. leucine (.3 g .per kg.) over 10 mins.
5: Fed 1.2 g. leucine (.155 g. per kg.) over 5 mins.
6: Case 2 fed 1.6 g. leucine (.15 g. per kg.) over 5 mins.
7: Case 3 fed 5.2 g. leucine (.083 g. per kg.) over 2 mins.
8: Case 2 fed 2.4 g. leucine (.225 g. per kg.) over 4 mins.
9: Case 1 fed 1.4 g. leucine (.15 g. per kg.) over 4 mins.
10: Case 4 fed 2.3 g. leucine (.15 g. per kg.) over 5 mins.
---: Non-sugar reducing substances of cases 2 and 3 determined simultaneously with the true blood sugars shown in curves 6 and 7.

reached the fasting level again after 60 to 90 minutes. Increasing the dose of leucine depressed the blood sugar level further and for a longer time (Figure 7, curves 6 and 8). The clinical effects of leucine varied: case 2 became drowsy and fell asleep 40 minutes after the test dose of 2.4 g. (Figure 7, curve 8); case 3 developed marked pallor 30 minutes after the test dose but did not complain of feeling unwell; in cases 1 and 4 severe convulsions occurred 45 and 54 minutes, respectively, after feeding the leucine, so that the experiment was terminated and glucose administered. No clinically observable effects were noted in any of the normal subjects. The dose of leucine fed was generally 0.15 g. per kg. body weight, *i.e.*, equal to the leucine contained in the standard test dose of casein; however, one of the normal subjects was fed twice this dose (Figure 7, curve 4), case 2 was fed one and one-half times this dose on one occasion (Figure 7, curve 8), and case 3 was fed only 0.083 g. per kg. (Figure 7, curve 7). He was fed the same dose as the other patients if one takes body weight to the power two-thirds as the basis of calculation (18) but on this basis the two adult normal subjects were fed about twice the standard dose of leucine.

Effect of isovaleric acid

Figure 8 shows the effect on the blood sugar of feeding commercial isovaleric acid to case 2 and to a normal subject. Commercial isovaleric acid contains only 55 to 60 per cent true isovaleric acid (β-methyl-n-butyric acid) the remainder being methylethylacetic acid (19, *cf.* 20). Hence case 2 (Figure 8, curve 2) was fed a dose of isovaleric acid corresponding to 0.085 g. of L-leucine per kg. body weight, while the normal subject (Figure 8, curve 2) was fed the equivalent of 0.15 g. L-leucine per kg.

True blood sugar and non-sugar reducing substances

The method briefly described here removes all known non-sugar reducing substances, but does not differentiate one sugar from another; it is a "true sugar" method but not a "true glucose" method (*cf.* 14). It will be described in greater

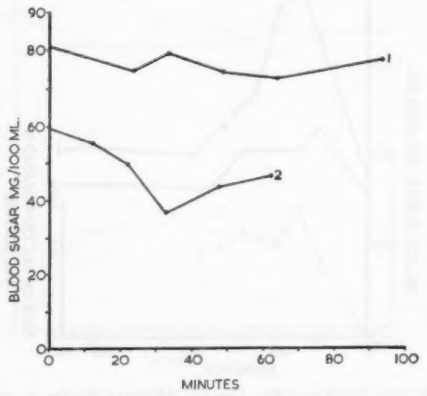


FIG. 8. BLOOD SUGAR AFTER BEING FED ISOVALERIC ACID

1: A normal subject fed 2.7 g. commercial isovaleric acid (.21 g. per kg. body weight) over 3 mins. 2: Case 2 fed 1.36 g. commercial isovaleric acid (.12 g. per kg.) over 2 mins.

detail elsewhere (21). With the exception of the glucose tolerance curves for cases 3 and 4 (Figure 1, curves 3 and 4), all the blood sugar values shown in Figures 1 to 8 were determined by this method.

In every specimen, in addition to the "true sugar," the "apparent sugar" was determined by omitting the treatment with Biodeminrolit G, the difference giving a value for non-sugar reducing substances (N.S.R.) remaining after tungstic acid precipitation. In the normal subjects the concentration of N.S.R. was equivalent to 13.4 ± 3.3 (S.D.) mg. of glucose per 100 ml. of blood (28 samples). The values were constant, within experimental error, for any given individual over the 90 minutes of a blood sugar curve, and were unaffected by feeding casein, glucose, leucine or isovaleric acid, but they varied slightly from day to day, and more markedly from individual to individual.

Most of the fasting specimens of blood from cases 1, 2, 3, and 4 had values for N.S.R. that fell within the normal range. However feeding leucine caused a marked fall in N.S.R. in cases 1, 2, and 3 (from 13.5 to 5.5 mg. "glucose" per 100 ml. blood for case 1; from 15.5 to 3.5 and from 14 to 3 for case 2; from 16.5 to 6.5 for case 3), the minimum value occurring 10 or 15 minutes later than the minimum for the true glucose (Figure 7) and the value returning to near normal in 90 minutes. In case 4 the fasting N.S.R. was equivalent to only 5 mg. glucose per 100 ml. blood and did not show any further fall on feeding leucine. Casein caused a similar but less marked drop in N.S.R. in cases 1, 2, and 4; case 3 was not fed casein. Gelatine, tyrosine and isovaleric acid had no effect on the N.S.R. in case 2. Feeding glucose had no effect at all on the N.S.R. in any of these cases nor did the injection of glucagon.

In another hypoglycemic child (not reported in detail, see Figure 4) the fasting N.S.R. was equivalent to only 2.5 mg. glucose per 100 ml. blood. On feeding casein the value dropped steadily to 1 mg. per 100 ml. at 30 and 45 minutes then rose to 2.5 mg. per 100 ml. at 60 minutes.

In case 1 most of the values for N.S.R. fell in the normal range, but when she was being fed breast milk, and was clinically rather better, a single preprandial specimen of blood at 2 p.m. had a "true sugar" content of 19 mg., and N.S.R. equivalent to 70 mg. glucose per 100 ml. Four hours later these values were: true sugar = 36, N.S.R. = 46 mg. glucose per 100 ml. blood. Next day, at 6 a.m. her fasting true sugar was 56, and N.S.R. = 16 mg. glucose per 100 ml. blood; the value for N.S.R. remained between 16 and 18 for the rest of the day.

On another day, when case 1 was being fed "Soylac," her blood glucose values were as follows:

Mg. glucose per 100 ml. blood

Time	6 a.m.	10 a.m.	11.40 a.m.	2 p.m.
True sugar	20	13	16	19
N.S.R.	28	13	6	2

By paper chromatography. While case 1 was on a cow's milk high protein diet, and suffering very frequent fits, her apparent blood sugar, determined by a modification of the method of Folin and Wu (15, 16), was 37 mg. per 100 ml., but by paper chromatography the glucose content was found to be 8.5 ± 1 mg. per 100 ml. Earlier the same day her blood glucose was 20 mg. per 100 ml. by paper chromatography, but her apparent blood sugar was 60 mg. per 100 ml. by the method of Folin and Wu. No sugar other than glucose was detected in either specimen.

There was never any detectable loss of glucose in the urine, either spontaneously or after feeding casein, in any of the patients. The other urinary sugars were normal. After case 4 had been fed glucose for a tolerance test, his urinary glucose reached 5 mg. per 100 ml., but this is still within normal limits.

The fasting blood concentrations of the different amino acids were normal in cases 1, 2, and 3 but somewhat higher than average in case 4 (two specimens), the relative proportions of the different amino acids were normal in all four. No explanation could be found, among the amino

TABLE I

Serum leucine content before and after a test dose of leucine

Subject	Case 1	Case 3	Normal	Normal
Weight, *Kg.*	9.1	62.5	86	68
Leucine fed, *g.*	1.4	5.2	12.8	10
Mg. leucine/100 ml. serum, fasting		1.2	1.2	
Mg. leucine/100 ml. serum, after the test dose	3.8	3.6	4	4.8
Time of second blood specimen, mins. after taking leucine	40	40	40	50

acids, for the abnormally high blood content of non-sugar reducing substances in case 1. After feeding leucine similar rises of the blood leucine content took place in two normal subjects, case 1 and case 4 (Table I), with no detectable change in the other amino acids.

In case 4 there was some degree of general amino aciduria, but in cases 1 and 2 the urinary amino acids were normal; they were not examined in case 3.

DISCUSSION

McQuarrie, Ulstrom, and Ziegler have referred to a special group with "infantile idiopathic hypoglycemosis" (22) in whom the age of onset of symptoms and the physical and laboratory findings are very similar to our present cases 1, 2, and 3.

In 1936, Conn (23) suggested the use of a high protein, low carbohydrate diet in the cases of idiopathic functional hypoglycemia. Subsequently this diet regimen has been tried in many cases of unexplained hypoglycemia. Talbot, Crawford, and Bailey's (24) case, prior to receiving alloxan, was tried on high protein, low carbohydrate feeds and it was noted that "during this period, the fasting blood sugar concentrations fell and the patient became so much worse that it became necessary to institute intravenous dextrose therapy." McQuarrie (6) also says of one of his patients on a similar diet that "her clinical state appeared to be getting worse with more frequent spells of irritability, staring and loss of consciousness."

The clinical observation that our patients (cases 1 and 2) had more fits when on a high protein diet led to an investigation of their blood sugar after feeding a test dose of casein. The result established a clear causative relation between the protein feeding and the hypoglycemia. To see whether this was a general effect of protein or amino acids, or whether some particular amino acid was responsible, one child was fed a test dose of gelatine, a protein with a very different amino acid composition from that of casein. Since there was a much smaller fall in the blood sugar concentration following gelatine it was probable that some particular amino acid or combination of amino acids was responsible for the hypoglycemia and a systematic search was started.

Tyrosine, which is almost absent from gelatine but composes 6.4 per cent of casein, was investigated first, but proved to have no effect on the blood sugar concentration. L-leucine was tried next and had a most dramatic effect. The extent of the hypoglycemic response varied with the dose of leucine; the effects of test doses of casein and gelatine could be fully accounted for by their leucine content. Other amino acids are being investigated but the results obtained with leucine seemed to merit publication at this time.

A sufficient number of normal individuals were tested with casein and leucine to indicate that these substances did not produce a fall in blood sugar under fasting conditions.

Very little work has been published in which human blood sugar concentrations have been measured at intervals after administering single amino acids. Manzini and Arullani (25) have reported that 50 to 80 mg. of leucine injected subcutaneously into normal fasting men causes a fall of about 25 per cent in blood sugar concentration; this may be contrasted with the results of our feeding experiments. Schenck (26) found that glycine in 40 to 50 g. oral doses caused a moderate reduction in the blood sugar concentrations of normal and diabetic adults (from 102 to 72 and from 256 to 161 mg. per 100 ml., respectively). Carballeira, Elrick, Mackenzie, and Browne (27) infused a mixture of ten amino acids (including

TABLE II

COMPOSITION OF MATERIALS FED TO CASES 1 AND 2 [1]

	CASEIN %	GELATIN %	HUMAN MILK. G. PER 100ML	COWS' MILK. G. PER 100ML	SOYLAC %[2]
PROTEIN	-	-	1·25	3·5	26
CARBOHYDRATE	-	-	7·5	4·8	53
LEUCINE	10·0	3·5	0·12	0·38	2·1
TYROSINE	6·4	0·5	0·06	0·21	1·0
PHENYLALANINE	6·3	2·3	0·07	0·19	1·4
GLYCINE	2·1	23·6	0·00	0·08	0·9
GLUTAMIC ACID	23·0	11·0	0·21	0·75	4·8
ASPARTIC ACID	7·0	6·2	0·10	0·25	1·0
ALANINE	3·3	8·2	0·03	0·11	0·86
ISOLEUCINE	7·5	1·7	0·08	0·26	1·6
VALINE	7·7	2·8	0·11	0·24	1·4
TRYPTOPHAN	1·3	0·0	0·02	0·05	0·36

[1] Compiled largely from Block and Bolling (49).

[2] A preparation of Soya flour with added carbohydrate. We are indebted to Soya Foods Ltd., Elstree, England, for some of the data.

L-leucine) intravenously into four normal fasting men and observed a slight drop (from 70 to 60 and from 76 to 60 mg. per 100 ml., respectively) in the blood sugar concentration in two of them 32 minutes after the mid-point of the infusion. A slight rise occurred in a third case and there was no change in the blood sugar of a fourth case.

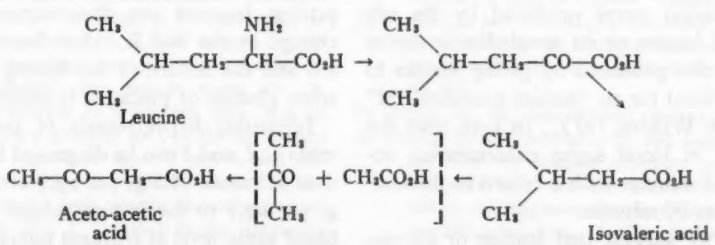
There is considerable evidence in the literature suggesting an inter-relationship between the blood levels of amino acids, glucose, insulin, and adrenaline. Feeding glucose to normal men causes a drop in blood amino acid levels, leucine being depressed more than the other amino acids (28, 29). Insulin (100 units upwards) also causes a drop in blood amino acids, leucine again being the most strongly affected (28, 30 *cf.* 31), but if glucose is continuously infused to keep the blood sugar above 80 mg. per 100 ml. after administering the insulin, there is no drop in blood amino acids (30, *cf.* 32).

Several workers have examined the effects of single amino acids, especially glycine, on the blood sugar of rabbits and dogs. A single oral, subcutaneous, or intravenous dose of glycine causes a rise in blood sugar level according to most workers (33–36); Folke Nord (33) and others have produced considerable evidence that glycine and some other amino acids act by causing the release of adrenaline. Schenck, on the other hand, found that glycine depressed the blood sugar level (26, 37), while others could find no effect (38, 39). According to some workers (40, 41) feeding glycine produces an initial hyperglycemia caused by the release of adrenaline, followed by prolonged hypoglycemia, caused by release of insulin in response to the hyperglycemia, though the liver plays some obscure part in the hypoglycemia (41). It is doubtful whether this explanation can account for all the results obtained by other workers. Manzini and Arullani (25, 35) found that leucine raised the blood sugar concentration of the rabbit but depressed that of man. The differences found by different workers for other amino acids may be partly explained by the use of different species.

A more relevant finding is that protein hydrolysates administered to humans simultaneously with glucose either intravenously (42) or orally (43) markedly reduce the height of the hyperglycemic curve that would result from administering the glucose alone, though the protein hydrolysate does not produce hypoglycemia in the fasting individual (43). (Compare Figure 5.) This may be compared with the finding of Chikano (39) that leucine, injected into rabbits, markedly reduced the hyperglycemia produced by injecting adrenaline or glucose, though it does not affect the fasting blood sugar level when given alone. However, Pollak (36) found that isovaleric acid had little or no effect on the glycemic curve in rabbits when fed with glucose.

For the four affected individuals considered here, but not for normal persons, leucine is a most potent drug, active when taken in comparatively low dosage. This effect must be distinguished from the toxic actions of the diamidines which cause hypoglycemia after initial hyperglycemia by depletion of liver glycogen stores (44, 45). Since leucine is always present in the blood it may be partly responsible for the low fasting blood sugar levels of these patients. It remained to be decided whether leucine itself caused the hypoglycemic response or whether it was converted at an abnormally high rate to an active intermediate metabolite, possibly one not formed in normal individuals.

According to Coon (46) L-leucine is metabolized according to the following scheme:



The keto-acid, formed from leucine by transamination, is not readily available. Isovaleric acid is split *in vivo* to a 3-carbon and a 2-carbon fragment, hypothetically acetone and acetic acid, each of which gives rise to aceto-acetic acid as end-product. Aceto-acetic acid cannot be the active substance since it is formed from tyrosine which has no hypoglycemic effect. Hence isovaleric acid remains the one readily available metabolite of leucine that might have a hypoglycemic effect. A test dose of commercial isovaleric acid produced as profound a hypoglycemia in case 2 as did an equivalent dose of leucine (allowing for the purity of the commercial acid). In a normal subject, however, a larger dose of isovaleric acid had no effect on blood sugar concentration. The most likely explanation is that isovaleric acid is the true hypoglycemic factor in these four patients and that leucine acts by virtue of its rapid conversion to isovaleric acid; a less likely alternative is that leucine and isovaleric acid have a similar effect, by virtue of their somewhat similar molecular structure, on some aspect of the blood sugar regulating mechanism. In any case we have no definite explanation of why these patients react to leucine and isovaleric acid while normal individuals do not.

There was no evidence of any abnormality of the liver in these patients and the response to adrenaline and to glucagon were normal. Nor were there any clinical stigmata of abnormal function of the pituitary, thyroid or adrenal gland. In case 4 there was hyperplasia of the islet cells of the pancreas and, in two specimens of blood, a high insulin level, but in the other three cases there was no direct evidence of hyperinsulinism. McQuarrie, Ulstrom, and Ziegler (22) have, however, mentioned that "infantile idiopathic hypoglycemia suggests a form of hyperinsulinism but without evidence of hypertrophy, hyperplasia, or tumour of the β-cells."

The blood sugar curve produced by the administration of leucine or its metabolite is almost identical with that produced by giving insulin to a normal individual for an "insulin tolerance test" as described by Wilkins (47). In both tests the maximal drop in blood sugar concentration occurs at 30 to 45 minutes with a return to the fasting level in 60 to 90 minutes.

Tentatively we suggest that leucine or its metabolite, isovaleric acid, acts by one of two mechanisms: either (a) it may enhance the effect of circulating insulin on intracellular carbohydrate metabolism, or (b) it may cause an increased secretion of insulin by the islets of Langerhans. It should be possible, using present techniques, to differentiate the two possible modes of action, and work is proceeding on this. In normal humans or animals leucine (alone or in mixtures) causes a decrease in the hyperglycemic curve that follows administration of glucose; this can be explained as an enhancement of the effect of the insulin released in response to the hyperglycemia or, perhaps as the release of additional insulin. The second possibility is rendered less likely but not completely excluded by leucine having no hypoglycemic effect in fasting normal humans or animals; the blood insulin level is presumably so low under these conditions that any enhancing effect of leucine would be undetectable. Of the four patients who showed a hypoglycemic response to leucine while fasting, one has been shown to have an abnormally high blood insulin level, again supporting possibility (a) but not excluding (b). However, the failure of a fifth hypoglycemic child to show a marked hypoglycemic response to casein (Figure 4) suggests the possibility of two types of hypoglycemia.

We have no explanation of the changes observed in blood non-sugar reducing substances (N.S.R.). There can however be no doubt that these substances (generally considered to be mainly glutathione and erogthioneine) are closely bound up with the blood glucose. In diabetics the N.S.R. level is often abnormal (21, 48). In three of the cases of hypoglycemia reported here the fasting N.S.R. has been, on occasion, well outside the normal range. Finally the fall and rise of the N.S.R. curve when leucine or casein is fed parallels, but lags a little behind, the fall and rise of the blood glucose curve. The most surprising features are the extraordinarily rapid change in the N.S.R. when leucine or casein is fed and the failure of the fasting N.S.R. to rise when glucose or glucagon is administered.

Idiopathic hypoglycemia of the type seen in cases 1, 2, and 3 can be diagnosed by feeding a test dose of casein (1.5 g. per kg.) or L-leucine (0.15 g. per kg.) to the fasting subject and noting the blood sugar level at frequent intervals for the next

ninety minutes. Other amino acids may well cause a fall in blood glucose and one of us (W.A.C.) is now investigating this possibility.

It would not be practical or even wise to attempt to restrict the protein intake of growing children but the results suggest that in this particular type of hypoglycemia high protein feeds should be avoided. We have suggested that small amounts of carbohydrate be given thirty to forty minutes after the ingestion of a feed to offset the effect of the protein in lowering the blood sugar. The natural course of the disease leads to less frequent fits as the child gets older but permanent mental deterioration will set in early in life if many hypoglycemic fits occur. The prognosis is good if acute hypoglycemic episodes are avoided by these means.

SUMMARY

Protein, leucine, or isovaleric acid, fed to three members of a family with spontaneous idiopathic hypoglycemia, caused a marked fall in the fasting true blood sugar level. They had a similar effect in a fourth child with hypoglycemia due to islet cell hyperplasia, but had no effect in normal fasting individuals. In another case of spontaneous idiopathic hypoglycemia casein had a less marked effect.

High protein feeds produced more fits and lower blood sugar values in the affected family than did a low protein diet.

The non-sugar reducing level was significantly outside the normal range in some blood samples from these patients and the apparent blood sugar level can be very misleading. The non-sugar reducing level fell nearly as fast as the true blood sugar level on feeding leucine or casein.

A casein test meal is advocated as a means of differentiating this type of hypoglycemia.

ACKNOWLEDGMENTS

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THE EFFECT OF ESTROGEN ON WATER AND ELECTROLYTE METABOLISM. I. THE NORMAL

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The possibility that estrogens might under certain circumstances influence water and electrolyte metabolism is suggested by several observations. For instance, estrogens are related chemically to the strongly sodium-retaining steroid desoxycorticosterone (1); the recurrent ankle edema observed in some women before menstruation was considered by Fluhmann (2) to be associated with an increase in blood estrogen levels; and the edema frequently found in late pregnancy occurs at a time when estrogen production is at its height (3).

These considerations led several workers to attempt to establish a direct relationship between water and salt retention on the one hand, and estrogenic activity on the other. Thorn and Engel (4) found sodium, chloride and water retention after a single injection of estrogen into male dogs. Later, the same group of workers (5), in a study of premenstrual fluid retention, found that decreased urinary excretion of salt and water was in two patients associated with an increased output of estrogenic material in the urine. Knowlton, Kenyon, Sandiford, Lotwin, and Fricker (6) found diminished urinary excretion of sodium following the daily administration of estradiol in one normal woman, but Sharpey-Schafer and Schrire (7), using twice the amount of estradiol in ten women, found no alteration in urinary volume.

As part of an investigation into the possible role of estrogens in the fluid retention of liver disease it became necessary to establish the effect of continued estrogen administration on water and electrolyte metabolism in the normal. Water and electrolyte balance studies were therefore carried out in eleven normal subjects, and the effect of daily administration of estrogen observed.

PROCEDURE

Eleven subjects without evidence of cardiac, hepatic, renal or other disease likely to affect water and electrolyte metabolism, were selected from the wards of The London Hospital. Five subjects were under treatment for peptic ulcer, during which time no medication other than magnesium trisilicate or aluminum hydroxide was given. Two subjects had sciatic nerve pain, one osteoarthritis of the hip, one torn medial meniscus of the knee, one hallux valgus, and in one a tuberculous pleural effusion had subsided three weeks previously.

Each subject underwent a water and electrolyte balance study, lasting approximately two weeks and divided into two periods—the first a "control period," and the second an "estrogen period," during which daily injections of estrogen were given. The subjects were given a normal diet, gauged in amount and composition to individual appetite, and remaining constant throughout the study in respect of Cl, Na, K, PO_4, water and calorie content. Diet composition was calculated from appropriate tables (8). Sample dietary analyses were not carried out. The balance diet was commenced 2 to 3 days before any observations were made. Since this diet corresponded closely in composition to previous intake in each case, no longer period for metabolic adjustment was considered necessary. In all subjects daily urinary output of water, Na and Cl was estimated. In eight subjects daily urinary K output and in six subjects daily urinary PO_4 output were measured. Fecal output of electrolytes and water were not determined. "Water balance" (Table I) was calculated by subtracting urinary volume from total water intake. Eight subjects were weighed daily in the fasting state, and in the same clothes. Cases 7, 20 and 21 were confined to bed, and weighing was not possible.

Blood analyses. Plasma concentrations of Cl and Na, the total serum protein and the venous hematocrit were determined on two separate days during the control period, and again on two separate days at the end of the estrogen period.

Extracellular fluid volume. In eight subjects an estimate of changes in extracellular fluid volume was obtained by determining the volume of distribution of a known quantity of thiocyanate. Exactly 20 ml. 4 per cent w/v NaCNS was injected intravenously, no food or drink having been taken for the previous twelve hours. Plasma samples were collected four and six hours later, and their thiocyanate concentration estimated. "Thiocyanate space" was calculated by linear extrapolation of these values to zero time. The space was estimated twice on separate days both during the control period and again at the end of the estrogen period. (Na injected for these determinations, about 10 mEq., was in addition to dietary Na intake).

Water tolerance test. After emptying the bladder, one liter of tap water was drunk in twenty minutes, no food

or drink having been taken for the previous twelve hours. The bladder was then emptied hourly for four hours, and the volume of urine measured. Two tests were carried out in the control period, and two at the end of the estrogen period, on separate days.

Estrogen. During the estrogen period, estradiol monobenzoate 10 mg. in oil was injected intramuscularly daily. This was equivalent to between 0.14 and 0.20 mg. per kg. body weight.

Chemical methods. Heparinized plasma was used for all determinations of plasma Na, Cl and CNS. Serum was used for total protein determinations. Plasma and urinary Na, and urinary K were estimated by the flame photometer of Holliday and Preedy (9), except in case 4 where the plasma and urine Na were estimated chemically as described by Aitken and Preedy (10). Plasma and urinary Cl was estimated by the method of Schales and Schales (11), urinary P by the method of Youngburg and Youngburg (12), total serum protein by the Kjeldahl method (13) and plasma CNS by the method of Bowler (14). The venous hematocrit was obtained by spinning heparinized blood in 10-cm. Wintrobe tubes for 20 minutes at 3,000 r.p.m., radius 15 cm.

Statistical analysis. Where daily measurements were obtained (*i.e.*, weight, water balance, urinary outputs of Cl, Na, K, PO_4), both individual cases and the group as a whole were statistically analyzed. Significant differences between mean values for control and estrogen periods were estimated by application of the t test, a value for P of 0.02 or less being taken as critical both here and in all succeeding tests. The significance of group mean differences between control and estrogen period means ($\bar{d}$) was similarly estimated.

Where less frequent observations were made (*i.e.*, plasma determinations of Cl, Na, serum protein, venous hematocrit, thiocyanate space) group analysis only was undertaken, the significance of group mean differences ($\bar{d}$) being estimated by the t test as above.

Weight measurements in cases 43 and 44 could not be so analyzed due to a steady gain in weight during both control and estrogen periods. Regression lines for both periods were therefore constructed. The significance of differences between control and estrogen regression lines were estimated by the t test.

RESULTS

Measurements of weight, "water balance," and urinary outputs of Na, Cl, K, PO_4 were obtained daily. Mean values for control and estrogen periods derived from these daily observations are given in Table I. Figures in brackets under each pair of means indicate the probability (P) that the difference between these means could have been due to chance. As "water balance" denotes the figure obtained when daily urinary volume is subtracted from total daily water intake, an increase in this figure implies retention of water.

Measurements of serum protein, venous hematocrit and thiocyanate space appear only in Table I, the figures representing means of two, or occasionally three, observations on separate days.

At the foot of Table I are given the group mean differences ($\bar{d}$) between control and estrogen periods for each class of observation (*e.g.*, urinary output of Cl, K, *etc.*) for the whole group, together with the value for P.

Weight. Group mean difference (without cases 43 and 44) was + 0.28 kg., $P = 0.1$, indicating no significant variation following the administration of estrogen. However, analysis of individual cases shows that there was in fact a small but statistically significant mean rise in weight in Nos. 4, 13, and 45 during the estrogen period. In addition, there was a significant alteration of the regression line slope in No. 44, indicating an increased rate of weight gain.

Water balance. No significant difference between control and estrogen periods was observed. Neither the group means nor the means in any of the eleven cases showed any significant change.

Urinary outputs. Chloride and sodium. Group Cl and Na means did not differ significantly between periods. There was no significant difference between means in any individual case, with the exception of No. 13, where the mean daily output of Cl increased by 36 mEq. ($P = 0.02$), but without any significant change in mean Na output.

Potassium. There was no significant difference between group means. Differences between means in the eight individual cases in which this measurement was made were likewise non-significant.

Phosphate. The was no significant difference between group means. However, in two cases out of the six in which this estimation was made (Nos. 21 and 76), there was a mean daily decrease in phosphate output of 13 and 17 mEq. respectively. These differences were significant ($P = 0.01$). In the remaining four cases the differences were non-significant.

Total serum protein level. No significant decrease in the group mean serum protein value was observed following the administration of estrogen ($\bar{d} = -0.26$ g. per 100 ml., $P = 0.1$). This estimation was done in seven cases.

Venous hematocrit. There was a significant decrease in the volume of packed cells, mean difference between control and estrogen period − 2.8

TABLE I

The effect of estrogen administration in the normal mean values for control periods (a) and estrogen periods (b) †

Case No.	Days in Control Period	Days in Estrogen Period	Weight (kg.)	Water Balance (ml.)	Daily Urinary Output: Chloride (mEq.)	Daily Urinary Output: Sodium (mEq.)	Daily Urinary Output: Potassium (mEq.)	Daily Urinary Output: Phosphate (mEq.)	Total Serum Protein (g./100 ml.)	Venous Hematocrit (%)	"Thiocyanate Space" (l.)
	(a)	(b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)
4	6	7	51.8 52.3 (0.02)	1000 820 (0.1)	139 149 (0.04)	113 135 (0.1)	- -	- -	- -	43.5 42.5	- -
7	4	9	- -	919 1046 (0.2)	113 118 (0.15)	134 120 (0.3)	- -	- -	6.1 6.5	48.0 42.5	13.6 15.2
13	8	9	46.0 46.5 (0.02)	811 702 (0.3)	136 172 (0.02)	162 171 (0.5)	- -	- -	- -	44.5 41.0	14.5 14.8
20	6	10	- -	950 986 (0.7)	166 146 (0.06)	132 111 (0.05)	98 94 (0.3)	82 81 (0.8)	- -	54 51	21.4 22.5
21	11	9	- -	790 851 (0.6)	87 77 (0.07)	75 64 (0.1)	54 43 (0.03)	43 30 (0.01)	7.4 6.6	- -	17.6 17.0
43	7	9	incr * (0.4)	1640 1194 (0.05)	152 154 (0.9)	148 151 (0.8)	95 111 (0.07)	54 56 (0.7)	6.4 6.0	46 42	21.5 21.6
44	8	8	incr * (0.01)	935 809 (0.3)	133 124 (0.4)	136 121 (0.2)	64 71 (0.1)	47 64 (0.08)	6.2 6.0	35.5 34.0	13.2 15.5
45	8	10	62.0 62.8 (0.01)	1464 1282 (0.2)	93 88 (0.6)	102 90 (0.03)	68 63 (0.3)	52 50 (0.5)	6.1 5.9	40.5 38	15.6 17.4
55	8	7	68.6 68.5 (0.8)	642 907 (0.8)	166 147 (0.08)	179 159 (0.15)	86 87 (0.8)	- -	6.7 6.7	51.5 50	20.8 20.8
74	8	8	70.5 70.5 (0.8)	950 1110 (0.25)	125 114 (0.07)	128 111 (0.07)	84 86 (0.5)	- -	- -	- -	- -
76	6	7	56.4 56.4 (0.5)	670 850 (0.07)	99 97 (0.7)	100 90 (0.15)	69 67 (0.7)	71 54 (0.01)	8.3 7.7	- -	- -
	Mean Difference		+0.28	-19	-2.8	-7.8	-1	-2.3	-0.26	-2.8	+0.83
	P		0.1	0.9	0.5	0.1	0.7	0.6	0.1	0.01	0.06

† Figures in brackets denote values for P relating only to the two means immediately above. An asterisk (*) indicates that the P value was derived from comparison of regression lines, mean values being omitted; "incr" indicates an increased and "decr" a decreased rate of weight gain. Mean differences for the whole group, with P values, are given at the foot of the table. Water balance = total water intake less urinary volume.

TABLE II

Water tolerance tests

Hourly urinary volume in ml. in (a) control period, (b) estrogen period. Each figure represents the mean of two or three tests on alternate days. $\bar{d}$ = group mean difference. P = probability derived from t test

Case No.	Subject	1st Hour (a)	1st Hour (b)	2nd Hour (a)	2nd Hour (b)	3rd Hour (a)	3rd Hour (b)	4th Hour (a)	4th Hour (b)	All hours All cases
4	H. L.	126	290	605	488	327	278	114	150	
7	P. D.	303	328	298	188	650	615	115	120	
13	A. W.	85	55	75	75	47	775	120	95	
20	W. D.	145	288	460	450	175	235	60	63	
21	E. E.	95	200	280	210	175	95	135	65	
$\bar{d}$		+87		−62		−15		−11		−1
P		0.3		0.3		0.8		0.9		<0.9

per cent, P = 0.01. This estimation was done in eight cases.

"Thiocyanate space." The difference observed between the control and estrogen period group means of the eight cases in which this estimation was made was of borderline significance ($\bar{d}$ = + 0.83 L., P = 0.06).

Plasma chloride and sodium levels. These observations are not included in Table I. No significant difference between control and estrogen periods was observed ($\bar{d}$ = 0 mEq. per L., P = 1, $\bar{d}$ = − 0.3 mEq. per L., P = 0.9, respectively).

Water tolerance tests. Details of the four hourly urinary volumes are given in Table II. Each figure represents the mean of two tests carried out on separate days. The tests were performed in five subjects. Mean differences for each hourly output between control and estrogen periods varied between + 87 and − 62 ml., none of which was significant. Mean difference for all hours and all cases was likewise non-significant.

For each subject daily urinary output figures for Cl and Na, together with those for water balance, were expressed as a percentage of the control pe-

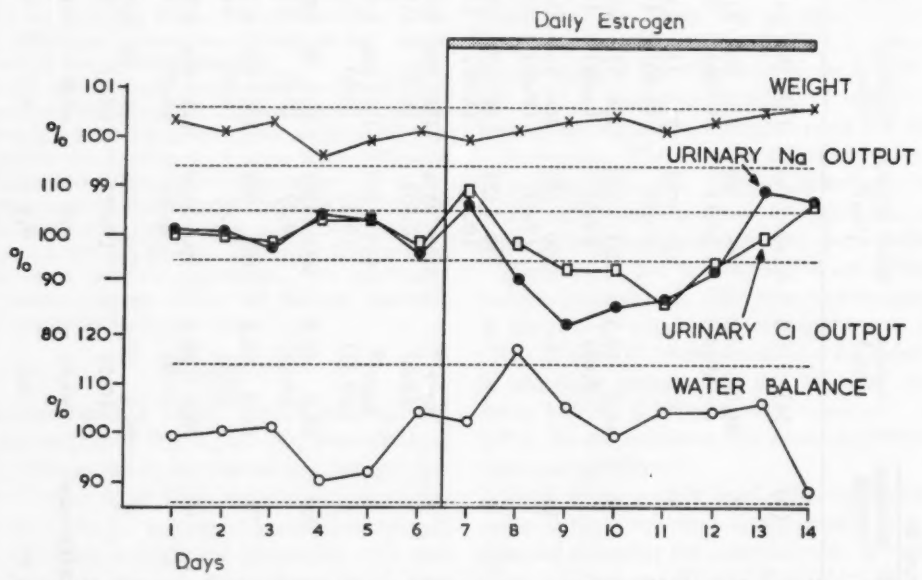

FIG. 1. THE EFFECT OF DAILY ESTROGEN ADMINISTRATION ON WEIGHT, URINARY Cl AND Na OUTPUT AND WATER BALANCE (TOTAL WATER INTAKE LESS URINARY OUTPUT) IN THE NORMAL

Control period: days 1 to 6, estrogen period: days 7 to 14. Each point represents the daily average for the group expressed as a percentage of the control period mean. Horizontal dotted lines denote 95 per cent confidence limits for the control period for each class of observation.

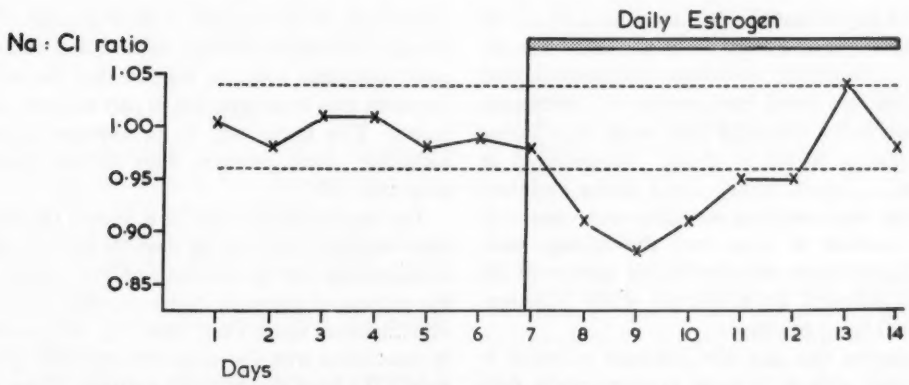

FIG. 2. THE EFFECT OF DAILY ESTROGEN ADMINISTRATION ON URINARY Na : Cl RATIO IN THE NORMAL
Control period: days 1 to 6, estrogen period: days 7 to 14. Each point represents the daily average for the group. Horizontal dotted lines denote 95 per cent confidence limits for the control period.

riod mean for that subject. The percentages for corresponding days were then averaged for all subjects and plotted graphically against days in Figure 1. Daily weights (cases 4, 13, 45, 55, 74, 76) were similarly treated. For convenience figures for the last six days only of the control period appear in the graphs.

Inspection of Figure 1 shows that following the administration of estrogen there was a systematic fall in Cl and Na output during the first four days, followed by a rise in the control level or above in the last four days. This was associated with a similar but inverse variation in water balance. That the water and electrolyte changes were closely related is shown by a Na/water balance correlation coefficient of 0.90, $P = 0.001$. Weight showed a slight tendency to rise after estrogen administration.

In Figure 2, the average daily urinary Na : Cl ratios for the group were plotted against days. There was a fall in the ratio, followed by a rise to control levels or above, during the estrogen period corresponding in time to the Na and Cl variations seen in Figure 1.

The statistical significance of these variations in Cl and Na output is indicated by the presence of at least four consecutive points below the 95 per cent confidence limits in Figures 1 and 2. As regards the weight observations, although the mean value in any one day did not lie outside the 95 per cent confidence limits, the fact that the last seven points in the estrogen period were above the control mean has a probability of occurring by chance of only $(\frac{1}{2})^7$, or 1 : 128. The highly significant negative correlation between Na output and water balance already mentioned, indicates that the variation observed in the latter was also significant.

Calculation of the cumulative balance of Cl, Na and water during the estrogen period (taking the mean of the control period as indicating the average output of each substance at equilibrium on the diet taken) showed that there was an average positive balance of Cl at the end of the estrogen period amounting to 20 mEq., and of Na amounting to 33 mEq. The Na : Cl ratio of these figures of 1 : 0.6 is that which would be expected, if all Cl and Na apparently retained had been stored as extracellular fluid (ECF). Calculated cumulative balance of water, however, appeared to indicate no change between periods, average balance figure at the end of the estrogen period being + 43 ml. only.

The average weight gain at the end of the estrogen period was 0.6 kg. (cases 43 and 44 were included by taking into account the difference between the regression lines). Of this, only 0.2 kg. could be accounted for by increased volume of extracellular fluid, if the latter were calculated from the average retention of Cl and Na given above.

DISCUSSION

Statistical analysis of the group as a whole (Table I) failed to show that estradiol administra-

tion had any sustained effect on weight, or on the urinary output of water, Na or Cl. However, although no sustained alteration in urinary Na and Cl output was found, the presence of a systematic variation in the output of these substances during the estrogen period is clearly demonstrated in Figure 1. Since dietary NaCl intake remained constant, and assuming no significant change in fecal excretion of these ions, the findings indicate that estrogen caused a limited storage of Na and Cl followed by a diuresis while injections were still being continued.

It appears that this NaCl storage occurred in association with an increase in extracellular fluid (ECF) volume, since there was no change in plasma Na and Cl concentrations, there was an increase in weight, and there were corresponding changes in water balance (Figure 1). There was in addition a significant decrease and increase in urinary Na : Cl ratio (Figure 2). The NaCl contents of a normal diet are roughly equivalent. This was reflected in the present series by an average urinary Na : Cl ratio of approximately 1 : 1 during the control period. Any increase in ECF volume under these conditions must be accompanied by a decrease in urinary Na : Cl ratio, such as is observed here.

Further evidence for an expansion of ECF is provided by the average increase in thiocyanate space, although this increase was of borderline significance only. The thiocyanate method for estimating ECF volume is open to various objections (15–17) (in common with many other methods), but is probably adequate when used to detect changes in the same subject under the conditions of the present study.

The diminution in serum protein concentration and venous hematocrit by almost the same percentage of the control mean (4.0 and 6.2, respectively) suggests a common influencing factor, that is, an increase in plasma volume of about 5 per cent. This is in agreement with the findings of other workers. Increased plasma volume has been found in both animals (18) and humans (19) after the injection of estradiol. It would appear, therefore, that the intravascular compartment takes part in the expansion of ECF following estradiol administration.

It is not certain that the observed increases in weight following estrogen can be entirely accounted for by an increase in ECF volume. The average cumulative balance figures, and those in some individual subjects, suggest that the weight increases may have been due in part to some other factor. This factor may be an increase in tissue formation, since estrogens have known anabolic properties (20).

The significant decrease in urinary PO_4 output after estrogen, observed in cases 21 and 76 might be accounted for by retention of PO_4 as part of the process of increased tissue formation. However, it seems more likely that PO_4 was retained in association with Ca, since Albright and Reifenstein (21) have shown that a positive calcium and phosphorus balance may be induced in patients with osteoporosis by estradiol administration. It is noteworthy that both our cases had a limb immobilized in plaster.

The absence of change in the result of water tolerance tests after estrogen is to be expected, since the tendency to store NaCl after estrogen administration in these subjects was both slight and transient, and daily urinary volume was not affected to any great degree.

Our results are in general agreement with those of Thorn and Engel (4) in dogs, and extend the observations of Knowlton, Kenyon, Sandiford, Lotwin, and Fricker (6) on one normal woman. The failure of Sharpey-Schafer and Schrire (7), using the same dosage of estradiol as ourselves, to find any alteration in urinary volume in their subjects was presumably due to the small changes involved. Urinary Na and Cl outputs (a more sensitive index) were not determined by them. However, Shapiro (22), using four to five times the dose of estradiol in normals, did observe a marked decrease in urinary volume followed by a diuresis on cessation of the estrogen. The difference between these results is presumably due to the dose employed.

The mode of action of estradiol in retaining salt and water is uncertain. The absence of any significant effect on urinary K excretion in dogs (4) or in man in the present series suggests that the action may not be via the adrenals. Moreover, Thorn and Engel (4) found that adrenalectomy did not affect the salt-retaining effect of estrogen in their dogs. The action of estradiol may therefore be directly in the kidney. Since it has been shown (23) that administered estradiol does not

affect the renal clearances of mannitol or of para-amino-hippuric acid, a direct action on the renal tubules may be involved.

SUMMARY

1. The effect of daily administration of estradiol on water and electrolyte metabolism has been studied in a group of 11 normal subjects.

2. A slight and transient decrease in urinary output of Cl, Na and water was observed, which was significant.

3. Plasma Cl and Na levels were not significantly affected.

4. There was no significant change in urinary K output in any of eight cases.

5. Urinary PO_4 output was not significantly changed in 4 out of 6 cases. In the two remaining cases, there was a small but significant decrease.

6. There was a decrease in total serum protein and venous hematocrit by approximately the same percentage amount, suggesting an increase in plasma volume.

7. Evidence is presented that the transient decrease in urinary Cl, Na and water output is associated with an increase in extracellular fluid volume.

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THE EFFECT OF ESTROGEN ON WATER AND ELECTROLYTE METABOLISM. II. HEPATIC DISEASE

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In chronic hepatic disease, the accumulation of fluid as ascites or edema is commonly observed (1). A similar tendency can also be demonstrated in acute hepatic disease, often in the absence of clinically detectable ascites or edema (2–4), suggesting that a common fluid-retaining factor may operate in both chronic and acute diseases.

Although diminished plasma colloidal osmotic pressure, resulting from lowered plasma albumen levels, has been put forward by some observers as the chief fluid-retaining factor in chronic liver disease (5–7) others have failed to find a constant correlation between the appearance of ascites and edema on the one hand and plasma albumen levels on the other (8, 9). Furthermore, the fluid retention of acute hepatic disease is not usually associated with abnormally low plasma albumen concentrations (2).

In search for other fluid-retaining factors, the possibility was suggested that in liver disease the hepatic inactivation of fluid-retaining hormones might be impaired, and from this point of view Ralli, Robson, Clarke, and Hoagland (10), investigated the role of the pituitary anti-diuretic hormone.

However, it seemed more probable that a primarily sodium-retaining hormone might be involved. Estrogens have been shown to have a salt-retaining action in animals (11), and there is much evidence to show that in animals these hormones are normally inactivated by the liver (12). Moreover, in human liver disease there are certain clinical features which are best explained on the basis of increased estrogen activity (13–15). These observations suggest that decreased inactivation of endogenous estrogen may occur in human liver disease, which may lead in turn to sodium-retention. Under these circumstances the administration of exogenous estrogen to cases of liver disease would be expected to result in far greater retention of sodium than in normal subjects (16).

The effect of the daily administration of estrogen on water and electrolyte metabolism has been studied in cases of human hepatic disease. To elucidate further the mechanism of the changes observed, the study was extended to include cases of obstructive jaundice, ascites without hepatic disease, and portal hypertension without hepatic disease.

CLINICAL DATA

Male and female cases were selected from the wards of The London Hospital. In no case was there evidence of cardiac or renal disease. In most cases, diagnosis was established by hepatic biopsy, at laparotomy or at subsequent post-mortem examination. Of the remainder only cases presenting typical clinical features were accepted.

Group 1—Hepatic cirrhosis with ascites (9 cases). The following categories were rejected: (a) Where accumulation of ascites was so rapid as to require paracentesis during the study. Only cases where ascites was accumulating or decreasing slowly were studied. (b) Where ascites could only be controlled by a low-Na diet. Control urinary Cl and Na levels under these circumstances were too low to enable the effect of estrogen to be adequately observed. (c) Where ascites had occurred only as a temporary episode complicating hematemesis. Two cases (Nos. 33 and 62) where ascites was about to develop were accepted, together with one (No. 56) where a chronic ascites had recently disappeared with treatment in hospital. One case (No. 62) was studied twice at different stages of the disease.

Group 2—Hepatic cirrhosis without ascites (12 cases). Cases in whom there was a past history of ascites only as a temporary episode complicating hematemesis were included. Two cases were studied twice at different stages of the disease (Nos. 16 and 22). Both early and advanced cases were included.

Group 3—Infective hepatitis (4 cases). All cases in this group (except No. 48) showed signs of recovery by the time estrogen was administered. Infective hepatitis occurred rarely during the period of these studies and only one case (No. 48) in the most severe category was available.

Group 4—Obstructive jaundice (5 cases). Only cases with chronic and apparently complete obstruction of the biliary passages were accepted. Cases of gall stones were excluded.

TABLE I

The effect of estrogen administration in cirrhosis with ascites †

Case No.	Days in Control Period	Days in Estrogen Period	Weight (kg.)		Water Balance (ml.)		Daily Urinary Output: Chloride (mEq)		Sodium (mEq)		Potassium (mEq)		Phosphate (mEq)		Total Serum Protein (g./100 ml.)		Venous Hematocrit (%)	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
1	8	12	incr * (0.01)		1150 (0.08)	1380	50 (0.06)	33	50 (0.01)	20	-	-	-	-	-	-	36	34
17	12	10	* (0.7)		2080 (0.4)	1910	50 (0.01)	25	20 (0.01)	10	-	-	-	-	-	-	41	36
24	10	12	incr * (0.01)		940 (0.01)	1410	158 (0.01)	94	146 (0.01)	68	56 (0.6)	58	71 (0.2)	78	6.6	6.2	37	36
28	9	9	incr * (0.01)		385 (0.01)	1110	144 (0.01)	51	156 (0.01)	35	55 (0.7)	53	54 (0.9)	54	7.1	6.7	43	41
33	8	9	55.8 (0.01)	57.5	930 (0.01)	1560	133 (0.02)	103	115 (0.01)	65	53 (0.2)	57	54 (0.2)	50	6.5	5.9	39	39
56	8	9	56.3 (0.01)	57.0	1180 (0.2)	1320	94 (0.2)	84	97 (0.2)	83	61 (0.01)	69	-	-	6.7	6.8	53	54
58	9	10	94.5 (0.01)	95.4	740 (0.1)	940	109 (0.06)	85	120 (0.05)	87	48 (0.7)	49	-	-	6.8	5.7	42	39
62	13	9	77.5 (0.6)	77.6	675 (0.7)	770	74 (0.08)	51	91 (0.02)	50	44 (0.4)	47	-	-	6.5	5.5	-	-
64	11	10	decr * (0.02)		1350 (0.07)	1570	55 (0.01)	19	49 (0.01)	10	46 (0.02)	35	52 (0.9)	52	-	-	-	-
	Mean difference		+0.85		+283		-36		-46		+1		+0.8		-0.57		-1.7	
	P		0.1		0.02		0.01		0.01		0.6		0.9		0.03		0.05	

† Mean values for control period (a) and estrogen period (b). Figures in brackets denote values for P relating only to the two means immediately above. An asterisk (*) indicates that the P values are derived from comparison of regression lines, mean values being omitted; "incr" indicates an increased, and "decr" a decreased rate of gain. Mean differences for the whole group with P values are given at the foot of the table. Water balance = daily total water intake less urinary output.

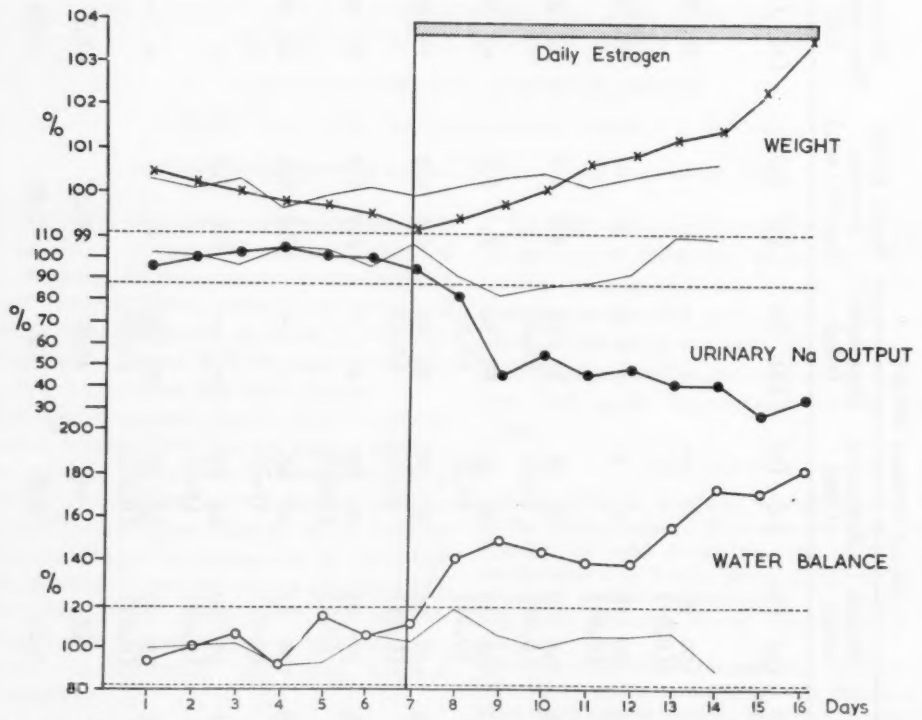

FIG. 1. THE EFFECT OF ESTROGEN ADMINISTRATION ON DAILY WEIGHT, URINARY SODIUM OUTPUT AND WATER BALANCE IN CIRRHOSIS WITH ASCITES

Control period, days 1 to 6; estrogen period, days 7 onwards. Each point represents the daily average for the group expressed as a percentage of the control period mean. Water balance = daily total water intake less urinary output. Horizontal interrupted lines denote the 95 per cent confidence limits for the control period for each class of observation. Results in the normal group are inserted for comparison (continuous line).

Group 5—Ascites without hepatic disease (3 cases). Cases were chosen where hepatic, cardiac or renal disease could be excluded. Cases were not accepted in which ascites was accumulating so rapidly that paracentesis might become necessary during the study, or in which ascites could only be controlled by a low Na diet. Two cases had carcinomatosis peritonei and one had tuberculous peritonitis.

Group 6—Portal hypertension without hepatic disease (2 cases). In the two cases in this group portal hypertension was due to splenic vein thrombosis. Liver function tests were normal, hepatic biopsy showed normal histology, and the liver appeared normal at laparotomy. There was no ascites.

The balance study, estradiol dosage, estimations, methods and presentation of results are as described in the previous paper (16) with the following exceptions. The "thiocyanate space" was not determined in cases with ascites, due to difficulty in obtaining an equilibrium CNS concentration between plasma and ascitic fluid. In statistical analysis an extended use was made of regression line comparisons, particularly in the analysis of weight trends. Values for P (derived from the t test) of between 0.03 and 0.05 were regarded as of "borderline significance." In calculating group mean differences for weight, cases where there was an obvious trend during the control period (marked by an asterisk in Tables I to IV) were omitted.

RESULTS

Cirrhosis with ascites (Table I)

Weight. The group mean difference between control and estrogen periods was calculated only from the four cases (Nos. 33, 56, 58, and 62) in which there was no obvious trend during the control period. In the remaining cases, regression lines were calculated for both periods. In all these subjects (except No. 17) a highly significant difference between control and estrogen regres-

sion lines was observed, the difference indicating an increased rate of weight gain during the estrogen period in case 1, and a slightly decreased rate of gain in case 64. In cases 24, and 28 there was a complete reversal of a downward trend following estrogen administration. In cases 33, 56, 58, and 62, the significance of differences between means could be tested. There was a significant increase of weight in cases 33, 56, and 58.

Water balance. There was a group mean difference between periods of + 283 ml. which was significant. In individual cases an increased mean figure was observed in all cases, except in No. 17, but the difference was of a magnitude to attain significance only in cases 24, 28, and 33.

Daily urinary outputs. Cl and Na. There was a group mean difference between periods of − 36 mEq. Cl and − 46 mEq. Na, each difference being highly significant. A significantly decreased mean output of Cl and Na during the estrogen period was observed in each subject, except in Nos. 56, 58, and 62. In No. 58 the decrease in both Cl and Na was of borderline significance, and in case 62 the decrease in Na was significant, but that in Cl was not. In case 56 no significant variation in either Cl or Na occurred during the estrogen period.

K. This estimation was performed in seven cases. There was no significant difference between periods as a group. In case 56, there was a small but significant increase in average output of K, and in case 64 a small but significant decrease.

PO_4. Phosphate output was studied in four cases. The group mean difference was not significant and there was no significant difference between period means in any individual case.

Total serum protein. There was a mean decrease of 0.57 g. per 100 ml., which was of borderline significance. This represents a mean decrease of 8 per cent of control values.

Venous hematocrit. A mean decrease of 1.7 per cent was observed in the seven cases in which this observation was made. The significance was borderline. This represents a mean decrease of 4 per cent of control values.

Plasma Cl and Na. No significant variation in concentration was observed following the administration of estrogen ($\bar{d} = -0.3$ mEq. per L. [P = 0.8] and + 0.7 mEq. per L. [P = 0.8], respectively).

Water tolerance test. Mean results for the five cases studied are given in Table IV. The hourly differences were either non-significant or of borderline significance, but the mean difference for all hours and all cases (− 67 ml.) was highly significant (P = 0.01).

In contrast to the normal findings there was a marked and sustained fall in daily average urinary Na output (Figure 1), accompanied by an

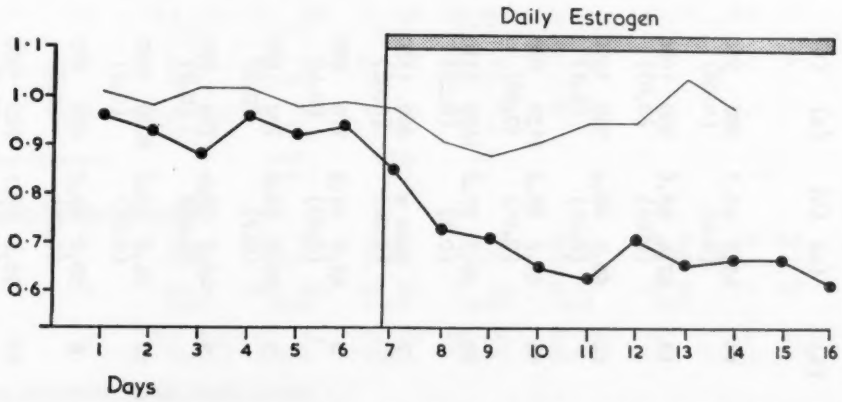

FIG. 2. THE EFFECT OF ESTROGEN ADMINISTRATION ON THE DAILY URINARY Na/Cl RATIO IN CIRRHOSIS WITH ASCITES

Control period, days 1 to 6; estrogen period, days 7 onwards. Each point represents the daily average for the group. Results in the normal group are inserted for comparison (continuous line).

TABLE II

The effect of estrogen administration in cirrhosis without ascites †

Case No.	Days in Control Period (a)	Days in Estrogen Period (b)	Weight (kg.) (a)	Weight (kg.) (b)	Water Balance (ml.) (a)	Water Balance (ml.) (b)	Daily Urinary Output: Chloride (mEq) (a)	Chloride (mEq) (b)	Sodium (mEq) (a)	Sodium (mEq) (b)	Potassium (mEq) (a)	Potassium (mEq) (b)	Total Serum Protein (g./100 ml.) (a)	Total Serum Protein (g./100 ml.) (b)	Venous Hematocrit (%) (a)	Venous Hematocrit (%) (b)	"Thiocyanate Space" (l.) (a)	"Thiocyanate Space" (l.) (b)
16	7	10	45.3 (0.8)	45.3	880 (0.08)	980	105 (0.08)	90	111 (0.02)	92	-	-	6.7	6.7	38	39	13.5	13.7
18	6	12	47.4 (0.01)	48.6	860 (0.03)	1050	69 (0.02)	50	62 (0.02)	49	-	-	6.3	5.5	40	38	15.6	17.8
19	8	12	68.1 (0.01)	68.4	960 (0.1)	1090	74 (0.2)	60	57 (0.2)	44	-	-	7.3	6.3	38	31	20.0	21.0
22	6	10	65.7 (0.01)	66.3	1150 (0.06)	890	90 (0.4)	99	79 (0.9)	80	51 (0.6)	53	7.2	7.2	38	37	18.0	19.4
39	8	10	56.7 (0.2)	57.0	1470 (0.09)	1110	130 (0.06)	115	135 (0.02)	110	63 (0.6)	62	7.2	7.0	33	32	24.5	25.5
50	7	7	deor m (0.06)		860 (0.05)	1130	106 (0.04)	90	105 (0.05)	84	41 (0.5)	40	6.7	5.7	33	32	-	-
63	8	8	41.6 (0.03)	41.2	710 (0.4)	805	108 (0.7)	105	120 (0.6)	114	71 (0.03)	65	7.0	6.7	36	34	-	-
65	12	9	62.5 (0.7)	62.5	790 (0.2)	680	110 (0.9)	111	106 (0.7)	110	64 (0.5)	66	6.6	6.1	-	-	-	-
70	10	9	49.7 (0.02)	50.4	730 (0.2)	870	88 (0.1)	69	89 (0.1)	67	63 (0.4)	66	6.8	6.8	-	-	-	-
71	7	10	64.8 (0.01)	66.2	1030 (0.9)	1030	93 (0.1)	82	89 (0.1)	77	56 (0.1)	51	6.0	5.6	-	-	-	-
72	8	8	50.1 (0.01)	49.7	655 (0.8)	675	106 (0.7)	102	117 (0.7)	106	28 (0.5)	27	6.4	6.5	-	-	-	-
73	10	10	58.5 (0.01)	58.1	925 (0.3)	1060	131 (0.2)	117	145 (0.05)	113	68 (0.05)	61	9.2	8.5	-	-	-	-
	Mean difference		+0.3		+28		-10		-14.1		-1.6		-0.4		-1.2		+1.16	
	P		0.1		0.7		0.01		0.01		0.9		0.01		0.2		0.03	

† See footnote, Table I.

equally sustained rise in water balance and in weight during the estrogen period. The extent of the 95 per cent confidence limits for the cirrhotic group indicates the high degree of significance of these findings. The figures for weight are necessarily cumulative, and, since there was a steady fall during the control period, the increase following estrogen administration should be measured from the last day of the control period and not from the control mean. The extent of weight gain is then seen to be far greater than in the normal.

Daily average urinary Na/Cl output ratios (Figure 2) decreased from a control mean of 0.94 to a sustained level of approximately 0.67 during estrogen administration, in contrast to normal values.

There was an average apparent retention during the estrogen period of 325 mEq. Na, 218 mEq. Cl,

TABLE III

The effect of estrogen administration in obstructive jaundice, infective hepatitis, ascites without hepatic disease and portal hypertension without hepatic disease †

Case No.	Days in Control Period (a)	Days in Estrogen Period (b)	Weight (kg.) (a) (b)	Water Balance (ml.) (a) (b)	Daily Urinary Output: Chloride (mEq) (a) (b)	Sodium (mEq) (a) (b)	Potassium (mEq) (a) (b)	Phosphate (mEq) (a) (b)	Total Serum Protein (g./100 ml.) (a) (b)	Venous Hematocrit (%) (a) (b)
OBSTRUCTIVE JAUNDICE										
3	7	7	51.2 49.4 (0.01)	650 820 (0.4)	155 114 (0.01)	165 147 (0.1)	- -	- -	- -	39 41
23	5	8	45.6 45.2 (0.7)	840 880 (0.6)	75 61 (0.1)	69 43 (0.03)	60 55 (0.2)	50 48 (0.7)	6.6 5.2	44 39
29	6	10	43.4 42.6 (0.01)	600 715 (0.1)	77 70 (0.2)	70 62 (0.4)	51 46 (0.04)	61 61 (0.9)	7.2 6.5	42 39
40	10	10	57.2 57.1 (0.6)	1450 1570 (0.5)	151 131 (0.2)	139 107 (0.03)	85 74 (0.05)	60 51 (0.7)	5.5 5.3	38 36
42	6	10	57.9 57.4 (0.3)	1165 1460 (0.09)	102 80 (0.01)	51 41 (0.1)	39 33 (0.06)	73 65 (0.2)	5.9 5.8	32 30
	Mean difference		-0.7	+148	-18.8	-20.8	-9.2	-3.8	-0.48	-3
	P		0.08	0.08	0.02	0.02	0.04	0.3	0.2	0.01
INFECTIVE HEPATITIS										
2	6	13	54.7 57.7 (0.01)	1085 1280 (0.2)	217 206 (0.4)	195 184 (0.2)	- -	- -	6.1 6.4	44 44
10	10	11	42.8 43.4 (0.01)	1230 1380 (0.01)	96 110 (0.3)	108 108 (0.7)	- -	- -	7.5 7.7	43 39
48	6	9	incr = (0.01)	215 995 (0.02)	249 167 (0.01)	260 168 (0.01)	70 76 (0.4)	- -	6.2 5.8	43 36
75	7	9	61.6 61.8 (0.1)	1210 1555 (0.05)	92 91 (0.8)	96 87 (0.2)	66 68 (0.5)	- -	8.3 7.7	- -
	Mean difference			+418	-25.4	-20			-0.18	-3.7
	P			0.04	0.3	0.9			0.5	0.2
ASCITES WITHOUT HEPATIC DISEASE										
25	10	10	incr = (0.01)	1200 1270 (0.5)	incr = (0.01)	incr = (0.01)	62 62 (0.9)	50 37 (0.01)	6.5 6.5	47 45
35	14	10	incr = (0.01)	1040 970 (0.4)	128 107 (0.09)	127 105 (0.06)	50 48 (0.6)	44 42 (0.7)	7.2 6.3	38 34
66	8	9	60.5 57.6 (0.01)	171 504 (0.01)	170 115 (0.01)	188 123 (0.01)	64 55 (0.03)	- -	- -	- -
PORTAL HYPERTENSION WITHOUT HEPATIC DISEASE										
14	8	10	57.4 58.0 (0.03)	830 980 (0.3)	158 125 (0.04)	153 133 (0.2)	- -	- -	6.0 6.0	41 39
49	8	9	65.9 66.5 (0.01)	890 1100 (0.1)	175 168 (0.4)	186 166 (0.2)	56 60 (0.5)	60 66 (0.1)	6.2 6.2	39 39

† See footnote, Table I.

and 1950 ml. water. There was an average gain of weight of 2.06 kg. These values are in approximately the expected proportion, if the changes observed were due to an increase in the volume of extracellular fluid (ECF). Since intake remained constant and there were no abnormal losses, these findings taken together indicate that the retention of Cl, Na, and water was in fact due to an increase in ECF volume.

Cirrhosis without ascites (Table II)

Weight. There was no significant difference between periods in the group as a whole. There was, however, a significant increase in weight in five individual cases (Nos. 18, 19, 22, 70, and 71), and a significant decrease in two (Nos. 72 and 73).

Water balance. The group mean difference of + 28 ml. was not significant. There was no significant difference between period means in any case, but in No. 18 a difference of + 190 ml. was of borderline significance.

Daily urinary outputs. Cl and Na. There was a small but significant difference between control and estrogen periods in both Cl and Na outputs, group mean differences being − 10.0 mEq. Cl and − 14.1 mEq. Na.

No significant difference was observed in individual cases, except in Nos. 16, 18, and 39. In cases 16 and 39 there was a small but significant decrease in Na output alone during the estrogen period; in No. 18 a significant decrease was observed in both Cl and Na.

TABLE IV

Water tolerance tests *

No. of subjects	Urinary volume (*ml.*) 1st hour	2nd hour	3rd hour	4th hour	All four hours
	Cirrhosis with ascites				
5	−80 (0.2)	−113 (0.04)	−51 (0.03)	−25 (0.06)	−67 (0.01)
	Cirrhosis without ascites				
5	−25 (0.7)	−100 (0.01)	−22 (0.6)	− 9 (0.8)	−39 (0.06)
	Obstructive jaundice				
5	+3 (0.3)	+58 (0.6)	−20 (0.7)	−58 (0.1)	−16 (0.6)

* Figures represent the group mean differences between control period and estrogen period tests (P figures are in parentheses).

K. There was no significant difference between periods as a group nor in any of the nine cases in which the estimation was done.

Total serum protein. There was a significant decrease, mean difference being − 0.4 g. per 100

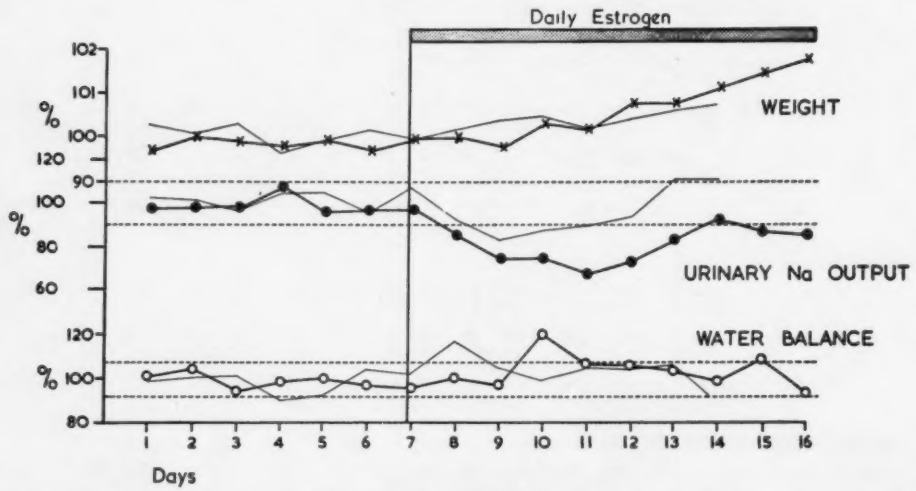

FIG. 3. THE EFFECT OF ESTROGEN ADMINISTRATION ON DAILY WEIGHT, URINARY SODIUM OUTPUT AND WATER BALANCE IN CIRRHOSIS WITHOUT ASCITES

See footnote, Figure 1.

ml. This represents a decrease of 5.5 per cent of control values.

Venous hematocrit. A mean decrease of 1.2 per cent was not significant. This estimation was done on seven cases. It represents a decrease of 3.0 per cent of control values.

"Thiocyanate space." This observation was made in five cases only. There was a mean increase of 1.16 L. which was of borderline significance.

Plasma Cl and Na levels. No significant change was observed in the level of either ion (group mean differences, + 0.2 mEq. per L. Cl and + 0.6 mEq. per L. Na, P = 0.8 in each case).

Water tolerance test (Table IV). This test was performed in five cases. The group mean difference between periods for all hours and all cases of − 39 ml. was not significant. Analysis of results by hours shows that there was a significant difference following estrogen administration only in the second hour ($\bar{d} = -100$ ml., P = 0.01).

Figure 3 indicates that there was no marked difference between the cirrhotic group and the normal in respect of average daily weight or water balance, but average daily Na outputs in the cirrhotic group were somewhat lower during the estrogen period. In addition, the Na output failed to return to the control mean in nine days, whereas in the normal group, values rose above the control mean in seven days. Daily average urinary Na/Cl ratios did not differ significantly from the normal.

There was an average apparent retention of 126 mEq. Na, 99 mEq. Cl, and 266 ml. water and an average weight gain of 0.74 kg. during the estrogen period. Na and Cl were retained in approximately the proportion expected, if retention were solely due to increased extracellular fluid volume, but the apparent retention of water and the increase in weight were not in the expected proportions.

Obstructive jaundice (Table III)

Weight. There was, as a group, no significant change during the estrogen period. Individual records show either no significant change (Nos. 23, 40, 42), or a small but significant decrease of weight during the estrogen period (Nos. 3 and 29). However, inspection of the individual records in these two cases indicated that a decreasing tendency was already evident during the last days of the control period, although there was no systematic tendency throughout the period as a whole.

Water balance. No significant variation occurred as a group during the estrogen period nor was there any significant variation in any individual case.

Daily urinary outputs. Cl and Na. There was a significant decrease in Cl and Na (group mean differences, − 18.8 mEq. Cl, and − 20.8 mEq. Na, P = 0.02 in each case).

In individual cases, there was a significant decrease in Cl output alone in cases 3 and 42, and a decrease in Na output alone of borderline significance in cases 23 and 40 (P = 0.03).

K. This estimation was done in four cases. A group mean difference of − 9.2 mEq. was of borderline significance (P = 0.04). A decrease of borderline significance also occurred in two individual subjects (Nos. 29 and 40).

PO_4. There was no significant variation as a group nor was there any significant difference in any of the four individual cases.

Venous hematocrit. There was a highly significant mean decrease in packed cell volume of 3 per cent (P = 0.01). This represents a decrease compared with control values of 7.5 per cent.

There was no significant change in either total serum protein levels or in thiocyanate space (mean difference for thiocyanate space + 1.9 L., P = 0.1).

Plasma Cl and Na. There was no significant change following estrogen administration (group mean difference + 0.2 mEq. per L. Cl and + 0.4 mEq. per L. Na, P = 0.9 in each case).

Water tolerance test (Table IV). The mean difference between control and estrogen periods for all cases and all hours was not significant. Analysis of hourly results indicated no significant difference between periods for any hour.

Figure 4 shows that Na output decreased well below the normal range, but rose above control levels by the end of the estrogen period. Water balance rose above the 95 per cent confidence limits, then returned to control values, the variation corresponding to that of Na output. Weight which had been falling steadily during the control period, rose during the estrogen period. Average daily urinary Na/Cl ratios did not however vary significantly between periods.

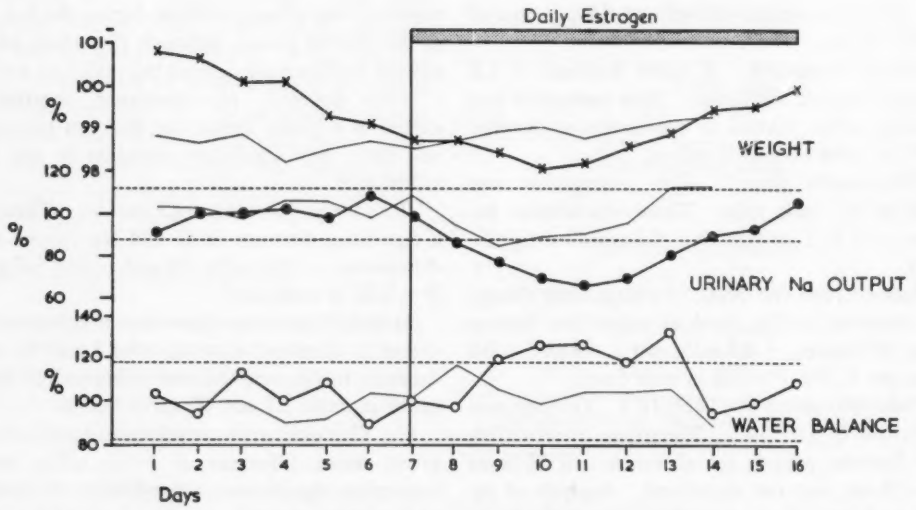

FIG. 4. THE EFFECT OF ESTROGEN ADMINISTRATION ON DAILY WEIGHT, URINARY SODIUM OUTPUT AND WATER BALANCE IN OBSTRUCTIVE JAUNDICE

See footnote, Figure 1.

There was an average apparent retention of 178 mEq. Cl and 167 mEq. Na, and 1360 ml. H_2O during the estrogen period. There was an average increase in weight (estimated from the last day of the control period) of 0.25 kg. These figures are not in the proportion expected if retention were due solely to an increase in ECF volume.

Infective hepatitis (Table III).

Weight. (Group analysis was not attempted.) There was a significant increase in weight during the estrogen period in cases 2, 10, and 48, being particularly marked in No. 2. In No. 48, the falling tendency during the control period was reversed following estrogen administration.

Water balance. There was a group mean difference of + 418 ml. between periods, which was of borderline significance ($P = 0.04$). In cases 10 and 48, there was a significant increase in water balance during the estrogen period. In case 75 the increase was of borderline significance ($P = 0.05$), and in case 2 non-significant.

Daily urinary outputs. Cl and Na. There was no significant group mean variation between periods in the output of either ion. In case 48, there was a significant fall in urinary Cl and Na output during the estrogen period. In the remaining cases there was no significant change.

No significant change was observed in urinary K output, nor in total serum protein, venous hematocrit and plasma Cl and Na levels. Water tolerance tests were carried out in three cases; there was no significant difference between periods.

In the four cases of infective hepatitis estrogen administration had no significant effect as a group on water balance or on urinary Cl and Na output. However, a significant decrease in urinary Na and Cl output, accompanied by a significant increase in weight and water balance, did occur in case 48. Here an NaCl and water diuresis was temporarily reversed on estrogen administration. The individual record showed that a second diuresis occurred when estrogen was discontinued.

Ascites without hepatic disease (Table III)

Group analysis was not carried out in these three cases. In case 25, there was a steady increase in weight during the control period due to the accumulation of ascites. Estrogen administration was accompanied by a slight but significant increase in the rate of weight gain. There was a progressive decrease in urinary Na and Cl output during the control period, comparison of regression lines indicating that the rate of decrease became significantly less during the estrogen period. A

significant mean decrease in urinary PO_4 output was observed during the estrogen period, and there was a reduction in venous hematocrit of 2 per cent.

In case 35, there was a steady decrease in weight during the control period, which was significantly reversed during the estrogen period ($P = 0.01$). No significant change was observed in urinary output of Cl, Na, K, or PO_4, but total serum protein decreased by 0.9 g. per 100 ml., and the venous hematocrit by 4 per cent. Case 66 was undergoing a slow spontaneous diuresis during the control period. The significant decrease in weight during the estrogen period was attributable to this factor, which also accounted for the significant diminution in water balance, and urinary Na and Cl output. A slight decrease in K output was of borderline significance.

Plasma Cl and Na levels were not significantly affected by estrogen administration in any of these cases. Water tolerance tests performed in cases 25 and 35 showed a mean difference between periods for both cases and all hours of -131 ml., which was of borderline significance ($P = 0.03$).

There was no indication in any of the three cases that estrogen caused any significant alteration in NaCl and water metabolism.

Portal hypertension without hepatic disease (Table III)

Group analyses were not carried out. In both cases studied there was an increase of weight following estrogen administration, significant in case 49 and of borderline significance in case 14. This was not reflected in any significant decrease in water balance or urinary output of Cl or Na. A mean difference of Cl only (-33 mEq.) in case 14 was of borderline significance. Urinary K and PO_4 outputs in case 49 were not significantly affected. Plasma Cl and Na and total serum protein levels were not significantly changed. A decrease in hematocrit of -2 per cent in case 14 was observed. The water tolerance test showed no significant change between periods in either case.

There was no indication that estrogen caused any significant retention of water or NaCl in either case.

DISCUSSION

The considerable and sustained retention of Cl, Na, and water observed in the group of cirrhosis with ascites following the administration of estrogen is in marked contrast with the effect of the same dosage in the normal (16). The changes in the cirrhotic group were apparent not only in the group as a whole (Table I, Figure 1 and 2) but also in each individual case, save one (No. 56) in which ascites had already disappeared following six weeks' hospital treatment.

In the group of cirrhosis without ascites, on the other hand, the effect of estrogen on NaCl and water metabolism did not differ markedly from the normal. However, although no significant change occurred in any individual case, save one (No. 18), a small but significant retention of Cl and Na was detected on group analysis (Table II, Figure 4), indicating that the difference between the two cirrhotic groups of the effect of estrogen administration is quantitative rather than qualitative.

The effect of estrogen on the group of infective hepatitis did not differ from the normal in three cases, but in the fourth (No. 48) estrogen administration was associated with a marked reversal of an existing NaCl and water diuresis similar to that observed in two cases (Nos. 24 and 28) in the group of cirrhosis with ascites. It is of interest that this (No. 48) was the only case of infective hepatitis in which recovery was not already evident by the time estrogen was administered, suggesting that NaCl and water retention might have been observed more often if more severe cases had been available.

The prominent increases in weight in the absence of NaCl retention, observed in some cases of infective hepatitis and cirrhosis without ascites after estrogen, may be attributable to increased tissue formation, as in the normal group (16).

The evidence (12) that, in sub-primates, the liver is the main site of estrogen inactivation is convincing. Although somewhat more controversial (17), the balance of evidence indicates that in humans also estrogens are chiefly inactivated in the liver (18, 19).

The marked NaCl and water storage observed in the present series of cirrhotics with ascites after estrogen administration might be accounted for by failure of a diseased liver to inactivate the hormone. However, the quantitative difference in NaCl and water retention observed between the two cirrhotic groups would then imply differing rates of hepatic estrogen inactivation. Before

this thesis can be accepted, various extrahepatic factors, which could account for the findings in the cirrhotics with ascites, must be excluded.

Increased abdominal tension (20) or congestion of the inferior vena cava (21) can cause, at least acutely, a decrease in renal clearance rates. Although the mechanism of renal excretion of estrogens is not known (22), it is possible that the presence of ascites might, by reducing renal clearances through one of the above mechanisms, delay the renal excretion of the estrogen. However, this possibility appears unlikely. No impairment of renal function was demonstrated in this group of cirrhotics with ascites by conventional renal function tests, and glomerular filtration rate and renal plasma flow have been shown to be in general normal in cirrhosis with ascites (23, 24) except possibly where ascites is accumulating rapidly (25). (Cases in the latter category were excluded from this study.) Furthermore, in this series marked NaCl and water retention following estrogen occurred before ascites had developed in case 33, and when ascites was absent in cases 18 and 48. Finally, no alteration in NaCl and water metabolism attributable to estrogen was observed when the hormone was administered to the group of ascites without hepatic disease.

It is also unlikely that portal hypertension was by itself an important factor in the differing response of the two cirrhotic groups, since firstly, portal hypertension, as judged by the demonstration of esophageal varices on barium swallow, was present in several of the group without ascites (notably Nos. 58 and 70, in whom the effect of estrogen administration did not differ significantly from normal), and secondly the response to estrogen administration in the group of portal hypertension without hepatic disease was within the normal range.

It appears therefore, that the differences observed between the two cirrhotic groups could reasonably be accounted for by differing rates of estrogen inactivation. This process may in turn be dependent on three intrahepatic factors.

There is good evidence that in animals estrogens are excreted in the bile (26, 27) and there is some evidence that biliary excretion may also occur in the human (28). If this pathway were important in the human, either for excretion in the feces, or as part of the process of hepatic inactivation, then either extrahepatic biliary obstruction or intrahepatic disorganization of the biliary tree would presumably result in high blood estrogen levels following estrogen administration. However, in the group of extrahepatic biliary obstruction (obstructive jaundice) in this series, although some significant retention of NaCl and water did occur (Table III) after estrogen administration, this was only of the same order as in the group of cirrhosis without ascites, and was moreover, transient (Figure 4). These observations indicate that failure of biliary excretion of estrogen due to a disorganization of the intrahepatic biliary tree is not mainly responsible for the NaCl and water retention observed in the group of cirrhosis with ascites following estrogen administration.

Although the process of hepatic inactivation must eventually depend on hepatocellular activity, an adequate hepatic circulation must also be essential. Since evidence from clinical observation, hepatic function studies and hepatic biopsy suggested that hepatocellular damage was rather more severe in our group of cirrhosis without ascites, it appears possible that the factor deciding the rate of hepatic inactivation of administered estrogen in this series, and so the degree of NaCl and water retention, may be an impairment of the hepatic circulation. Estimated hepatic blood flow is known to be reduced in cirrhosis of the liver (29), but the discrete effects of ascites have not been studied.

Our observations support the view that the action of estrogens may be a factor in the fluid retention of liver disease, since if exogenous estrogen can produce NaCl and water retention, then endogenous estrogen would be expected to have the same effect, provided that adequate quantities of the hormone were being produced. The findings of increased urinary estrogenic material on bioassay in both chronic (30, 31) and acute (32) hepatic disease indicates that estrogen production in these conditions is in fact substantial.

SUMMARY

1. Daily administration of 10 mg. estradiol benzoate caused sustained retention of NaCl and water in eight of nine cases of hepatic cirrhosis with ascites.

2. In hepatic cirrhosis without ascites the same dose of estradiol caused NaCl and water retention only slightly greater than in the normal, in eleven of twelve cases.

3. The effect of estradiol in five cases of jaundice due to extrahepatic biliary obstruction was similar to that in cirrhosis without ascites.

4. In a group of four cases of infective hepatitis estradiol caused significant NaCl and water retention in one only.

5. In three cases of ascites without hepatic disease and two cases of portal hypertension without hepatic disease, no significant NaCl and water retention attributable to estradiol administration was demonstrated.

6. Evidence is put forward suggesting that the NaCl-retaining effect of estradiol in the group of cirrhosis with ascites is due to decreased hepatic inactivation of the hormone. This decreased inactivation may in turn be dependent mainly on a disturbance of the hepatic circulation, rather than on hepatocellular damage.

7. The above findings indicate that increased estrogen activity may be a factor in the fluid retention of liver disease.

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THE EFFECT OF ESTROGEN ON WATER AND ELECTROLYTE METABOLISM. III. CARDIAC AND RENAL DISEASE

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If the sodium chloride and water retention observed in cirrhosis with ascites after estrogen administration (1) were due to delayed hepatic inactivation of the hormone, and if this were in turn dependent on an impairment of the hepatic circulation rather than on hepatocellular damage, then a similar retention of NaCl and water after estrogen would be expected in conditions where the hepatic circulation was prominently affected, but hepatocellular damage minimal.

Early cases of constrictive pericarditis and cardiac failure from other causes were selected as fitting most nearly into the above category, and they, together with more advanced cases, were submitted to water and electrolyte balance studies, during which the effects of estrogen administration were noted. Two cases of venous obstruction without cardiac failure were similarly studied for comparison.

Since any changes in NaCl and water metabolism observed in the above groups might be attributed equally well to delayed renal clearance as to decreased hepatic inactivation of the hormone, the effect of estrogen administration in water and electrolyte metabolism was also studied in primary renal disease, and for this purpose a group of cases with the nephrotic syndrome was selected, and studied as above. Two cases of low plasma albumin edema without renal disease were similarly studied for comparison.

CLINICAL MATERIAL

1. *Constrictive pericarditis* (*7 cases*). The cases selected fall into two groups:

(a) Four cases without symptoms, in whom the disease was discovered at routine medical examination for the Armed Forces, or for other purposes (Nos. 8, 9, 52 and 59). There was no detectable ascites in these cases. Liver function tests were normal, except for a raised serum bilirubin (up to 2.5 mg. per 100 ml.), or a slight excess of urinary urobilinogen in some cases. Liver biopsies in cases 52 and 59 showed minimal focal fibrosis only and no cellular damage. These subjects were ambulant for part of the day during the study.

(b) Three cases with definite symptoms referable to the disease (Nos. 46, 53 and 67). Two cases (Nos. 53 and 67) had had symptoms for several years, and one case (No. 53) had had a partial pericardectomy five years previously, with little relief. One case (No. 53) had ascites at the time of the study, and two cases (Nos. 46 and 67) had a recent history of ascites, but no detectable ascites at the time of the study. Liver function tests were normal except for raised plasma bilirubin levels (up to 2.9 mg. per 100 ml.). Liver biopsy in case 46 showed slight focal fibrosis and no cellular damage, and in case 53 extensive dense fibrosis without significant parenchymal change. Cases 46 and 67 were partly ambulant, and case 53 at bed rest during the study. Blood urea levels were normal in each group.

2. *Cardiac failure* (*5 cases*). Cases in this group were suffering from cardiac disease other than constrictive pericarditis—valvular disease in three cases (Nos. 12, 31 and 32) and of uncertain origin (?arteriosclerotic) in two cases (15 and 47). One case was in her first attack of failure (No. 32), one in her second attack (No. 47) and the remainder had had features referable to cardiac failure for 2 to 5 years. Liver function tests were normal in all cases except No. 12, where the plasma bilirubin was raised to 2.3 mg. per 100 ml. Blood urea levels were normal.

3. *Venous obstruction* (*2 cases*). One case in this group (No. 28) had superior vena cava obstruction due to a mediastinal gumma (shown at postmortem). There was constant marked edema of the upper part of the body. The other case (No. 68) the inferior vena cava was thrombosed, and there was marked edema of the lower half of the body, which rapidly subsided with rest in bed, accompanied by a NaCl and water diuresis. There was no evidence of cardiac, renal or hepatic disease in either case.

4. *Nephrotic syndrome* (*5 cases*). In four of the cases in this group (Nos. 30, 37, 51, 57) the syndrome was caused by chronic glomerulo-nephritis and in the fifth (No. 54) by primary renal amyloid disease. There was no evidence of cardiac or hepatic disease. Duration of the edema was 1 to 18 months, except in case 30 where it had been present for 5 to 6 years. There was no arterial hypertension in any case. Blood urea levels were either normal or somewhat raised. Ascites was present in two cases (No. 37 and 51). Conventional renal function tests either yielded normal results, or indicated some impairment of function.

5. *Low plasma albumin edema* (*2 cases*). One case (No. 69) in this group had chronic edema of the legs, associated with a constant plasma albumin of between 2.4 and 1.7 g. per 100 ml. over a period of years, which

TABLE I

The effect of estrogen administration in constrictive pericarditis †

Case No.	Days in Control Period	Days in Estrogen Period	Weight (kg.)		Water Balance (ml.)		Daily Urinary Output: Chloride (mEq)		Daily Urinary Output: Sodium (mEq)		Daily Urinary Output: Potassium (mEq)		Total Serum Protein (g./100 ml.)		Venous Hematocrit (%)	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
8	8	7	incr * (0.01)		850 (0.01)	1415	197 (0.01)	142	201 (0.01)	121	-	-	6.6	6.9	49	48
9	8	9	66.4 (0.01)	68.8	1270 (0.6)	1270	204 (0.2)	172	186 (0.2)	170	-	-	6.6	7.0	50	45
46	8	11	54.6 (0.02)	55.8	780 (0.7)	840	122 (0.01)	81	126 (0.01)	72	67 (0.5)	65	6.7	6.2	50	45
52	9	9	57.9 (0.01)	59.0	1090 (0.09)	1260	127 (0.01)	83	123 (0.01)	77	85 (0.04)	93	7.6	7.3	48	44
53	8	11	incr * (0.01)		23 (0.01)	935	217 (0.01)	97	240 (0.01)	98	55 (0.6)	54	6.8	6.6	36	39
59	8	9	incr * (0.01)		790 (0.01)	1280	137 (0.01)	102	141 (0.01)	89	68 (0.01)	61	8.2	8.0	47	45
67	9	9	incr * (0.01)		1310 (0.02)	1450	71 (0.5)	65	58 (0.4)	49	69 (0.3)	73	7.5	7.6	48	47
		Mean Difference			+334		-4.8		-57		+0.4		-0.06		-2.1	
		P			0.04		0.02		0.02		0.6		0.5		0.1	

† Mean values for control period (a) and estrogen period (b). Figures in brackets denote values for P relating only to the two means immediately above. An asterisk (*) indicates that the P values are derived from comparison of regression lines, mean values being omitted; "incr" indicates an increased, and "decr" a decreased rate of gain. Mean differences for the whole group with P values are given at the foot of the table. Water balance = daily total water intake less urinary output.

was unexplained. In the other case (No. 77), the low plasma albumin levels were due to inadequate dietary intake of protein, partially remedied during the course of the study by a high protein diet.

The balance study, estradiol dosage, estimation and methods and presentation of results are as previously described (2).

RESULTS

Constrictive pericarditis (Table I)

Weight. Group mean difference between control and estrogen periods was not calculated due to the proportionately large number of weight trends during the control period. There was a significant weight gain following estrogen administration in each of the 7 cases studied.

Water balance. There was a group mean difference between periods of + 334 ml. which was of borderline significance (P = 0.04). There was a significant mean increase in water balance in 4 of the 7 cases studied.

Daily urinary output. Cl and Na. There was a significant mean decrease in Cl and Na output

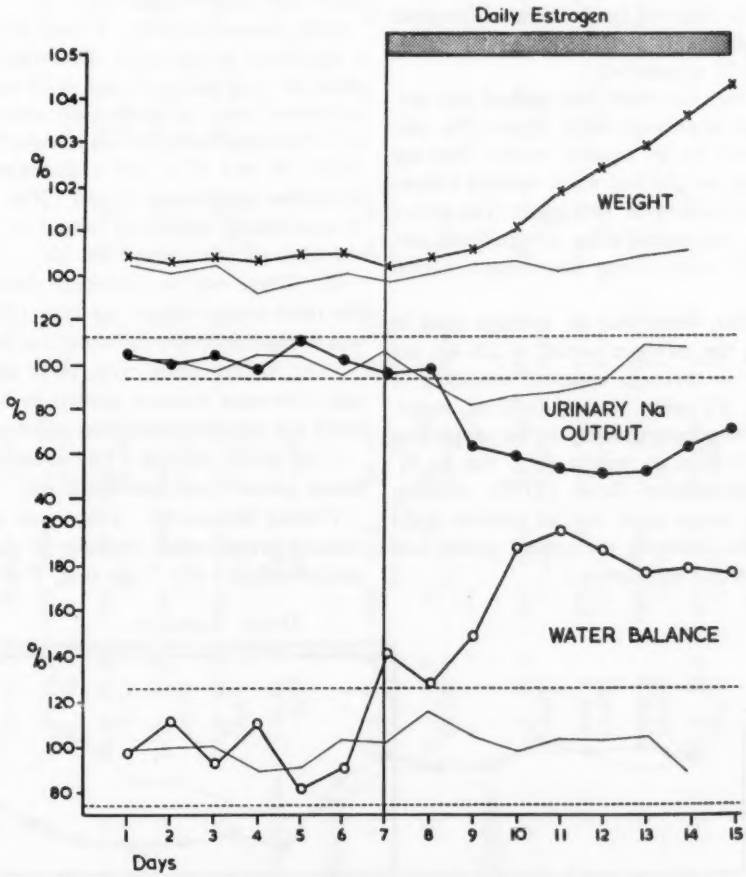

FIG. 1. THE EFFECT OF ESTROGEN ADMINISTRATION ON DAILY WEIGHT, URINARY SODIUM OUTPUT AND WATER BALANCE IN CONSTRICTIVE PERICARDITIS

Control period, days 1 to 6; estrogen period, days 7 onwards. Each point represents the daily average for the group expressed as a percentage of the control period mean. Water balance = daily total water intake less urinary output. Horizontal interrupted lines denote the 95 per cent confidence limits for the control period for each class of observation. Results in the normal group are inserted for comparison (continuous line).

following estrogen, group mean difference being − 48 mEq. and − 57 mEq. respectively. A significant decrease in Cl and Na output occurred in 5 of the 7 cases.

K. Estrogen administration had no effect on K output as a group. There was a small but significant decrease in case No. 59, and a small increase in case 52, which was of borderline significance.

Total serum protein and venous hematocrit were not significantly affected.

Plasma Cl and Na. No significant difference in these levels was observed (group mean differences − 0.4 mEq. per L. [P = 0.8], and + 0.4 mEq. per L. [P = 0.9] respectively).

Figure 1 shows that there is a marked and sustained decrease in average daily urinary Na output accompanied by an equally marked increase in average daily weight and water balance following the administration of estrogen. The above changes were accompanied by a significant decrease in Na/Cl ratio during the estrogen period (Figure 2).

By calculation, there was an average gain in weight during the estrogen period of 2.6 kg. accompanied by an average apparent retention of 315 mEq. Cl., 377 mEq. Na and 1970 ml. water. These figures are approximately in the proportion expected if the gain in weight were due to increase in extracellular fluid (ECF) volume. (Case No. 53 where there was an obvious NaCl and water diuresis during the control period was not included in this calculation.)

Cardiac failure (*Table II*)

Weight. There was a significant group mean difference between periods of + 0.82 kg. In individual cases, there was a significant increase in weight in two cases (Nos. 31 and 47), and an increase of borderline significance in one case (No. 12).

Water balance. There was a group mean increase following the administration of estrogen of 256 ml. which was of borderline significance. A significant increase was observed in two individual cases only (Nos. 12 and 31).

Daily urinary outputs. Cl and Na. There was a significant group mean difference between periods of − 23 mEq. Cl and − 28 mEq. Na. In individual cases, a significantly decreased output of Cl following estrogen was observed in two cases (Nos. 31 and 47), and a decreased output of borderline significance in two (Nos. 12 and 32). A significantly decreased output of Na was observed in all cases except No. 15.

K. There was no significant change in two of the three cases, and in one case (No. 32) there was a small increase of borderline significance.

PO_4. Of the three cases, there was no significant difference between periods in No. 31, but a small but significant decrease in cases 32 and 47.

Total serum protein. The mean difference between periods was non-significant.

Venous hematocrit. There was a highly significant group mean decrease in the volume of packed cells ($\bar{d}$ = − 5 per cent, P = 0.02). This

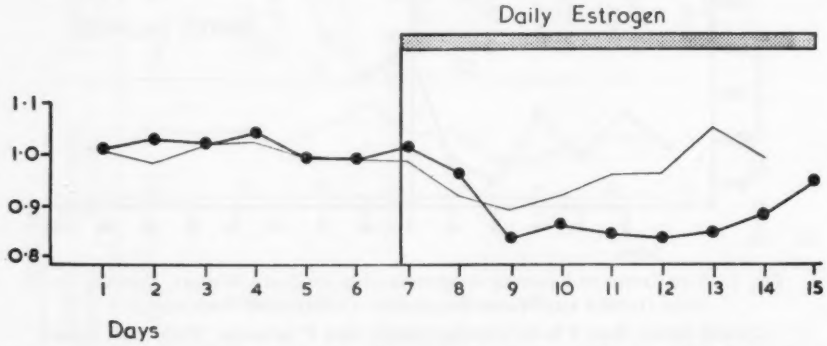

FIG. 2. THE EFFECT OF ESTROGEN ADMINISTRATION ON THE DAILY URINARY Na/Cl RATIO IN CONSTRICTIVE PERICARDITIS

Control period, days 1 to 6; estrogen period, days 7 onwards. Each point represents the daily average for the group. Results in the normal group are inserted for comparison (continuous line).

TABLE II

TABLE II

The effect of estrogen administration in cardiac failure, venous obstruction, nephrotic syndrome and low plasma protein edema †

Case No.	Days in Control Period	Days in Estrogen Period	Weight (kg.)	Water Balance (ml.)	Daily Urinary Output: Chloride (mEq)	Daily Urinary Output: Sodium (mEq)	Daily Urinary Output: Potassium (mEq)	Daily Urinary Output: Phosphate (mEq)	Total Serum Protein (g./100 ml.)	Venous Hematocrit (%)	"Thiocyanate Space" (l.)
	(a)	(b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)	(a) (b)
CARDIAC FAILURE											
12	13	10	65.7 66.5 (0.05)	995 1405 (0.02)	129 102 (0.04)	112 93 (0.01)	- -	- -	- -	50 44	21.1 22.4
15	8	10	54.2 54.8 (0.09)	1060 1070 (0.9)	114 98 (0.3)	116 96 (0.2)	- -	- -	6.2 6.8	48 41	21.2 23.0
31	11	12	52.5 54.0 (0.01)	530 900 (0.01)	104 83 (0.02)	114 86 (0.01)	50 49 (0.7)	26 23 (0.4)	6.2 6.2	51 43	19.8 23.3
32	7	10	50.8 51.2 (0.2)	850 960 (0.3)	104 86 (0.04)	105 79 (0.02)	59 66 (0.05)	46 39 (0.01)	7.7 7.1	46 44	19.0 22.0
47	7	8	50.5 51.3 (0.01)	640 920 (0.1)	154 121 (0.02)	166 120 (0.02)	51 48 (0.5)	60 46 (0.01)	6.4 6.0	37 35	17.2 20.2
	Mean difference		+0.82	+256	-23	-28			-0.1	-5	+2.5
	P		0.02	0.03	0.01	0.01			0.7	0.02	0.01
VENOUS OBSTRUCTION											
26	7	10	72.4 73.4 (0.01)	1135 915 (0.1)	99 111 (0.3)	96 118 (0.3)	58 45 (0.03)	- -	6.1 5.8	- -	- -
68	4	9	79.4 76.7 (0.01)	65 915 (0.01)	238 136 (0.01)	264 131 (0.01)	67 73 (0.3)	- -	5.9 5.9	- -	- -
NEPHROTIC SYNDROME											
30	10	14	49.5 51.7 (0.01)	830 1090 (0.02)	93 65 (0.01)	86 59 (0.01)	47 44 (0.4)	36 29 (0.01)	4.3 3.7	38 34	- -
37	7	9	decr ≡ (0.01)	770 730 (0.7)	200 195 (0.6)	191 201 (0.4)	58 68 (0.1)	64 58 (0.3)	3.1 2.8	34 31	36.5 37.5
51	9	9	decr ≡ (0.01)	1090 1080 (0.9)	117 117 (0.9)	107 107 (0.9)	69 67 (0.6)	- -	4.4 3.9	36 32	21.5 22.0
54	8	9	85.3 85.6 (0.09)	710 830 (0.2)	119 100 (0.05)	114 98 (0.1)	74 75 (0.9)	- -	3.9 4.0	52 45	31.9 32.9
57	9	9	decr ≡ (0.01)	1320 1030 (0.03)	61 81 (0.07)	52 80 (0.04)	72 79 (0.2)	- -	4.1 4.1	46 43	- -
	Mean Difference			-12	-1	-6	-0.2		-0.26	-4	+0.8
	P			0.5	0.9	0.5	0.8		0.2	0.01	
LOW PLASMA ALBUMEN EDEMA											
69	9	8	65.5 65.8 (0.05)	995 1275 (0.01)	118 123 (0.7)	129 120 (0.5)	58 67 (0.05)	- -	3.3 3.4	- -	- -
77	9	8	69.9 70.8 (0.01)	990 1350 (0.1)	158 124 (0.1)	168 124 (0.05)	77 76 (0.9)	- -	5.8 6.8	- -	- -

† See footnote, Table I.

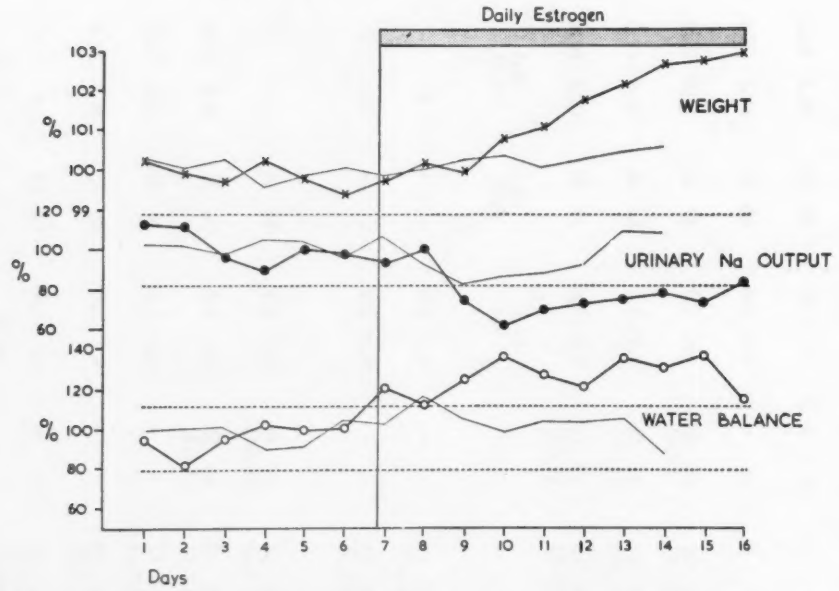

FIG. 3. THE EFFECT OF ESTROGEN ADMINISTRATION ON DAILY WEIGHT, URINARY SODIUM OUTPUT AND WATER BALANCE IN CARDIAC FAILURE

See footnote, Figure 1.

represents a mean decrease below control values of approximately 11 per cent.

Plasma Cl and Na. There was no significant difference between periods ($\bar{d} = +0.4$ mEq. per L. in each case, $P = 0.7$, and 0.8 respectively).

Thiocyanate space. There was a group mean increase following estrogen of + 2.5 L. which was significant.

Figure 3 shows the significant increase in average weight and water balance, and decrease in average urinary Na output which occurred during the estrogen period.

By calculation there was an average weight gain during the estrogen period of 2.3 kg. and an apparent retention of 221 mEq. Cl., 282 mEq. Na and 2170 ml. water. The thiocyanate space was significantly increased by a mean of 2.5 L. These figures are in the proportion expected if the gain in weight were chiefly due to an increase in ECF volume.

Venous obstruction (Table II)

Group analysis was not attempted. In case 26, there was a significant increase in weight during the estrogen period. This was unaccompanied by any significant difference between periods in respect of water balance, or of urinary Cl, Na or K output. The decrease in plasma protein concentration was of the same order as in the normal group. The small differences observed in plasma Cl and Na concentration were within the experimental error of the methods.

In case 68, a NaCl and water diuresis occurred spontaneously at bed rest. Weight showed a rapid decline during the control period, and levelled off during the estrogen period. The significant differences between periods observed in water balance and urinary Cl and Na outputs are due to the gradual cessation of diuresis during the estrogen period. There was no significant change in urinary K output, nor in total serum protein or plasma Cl and Na levels. The failure of estrogen administration to affect significantly the NaCl and water diuresis in this case is in contrast to the effect on case 53 (constrictive pericarditis), and on other cases (1) where a similar diuresis was reversed by estrogen.

Nephrotic syndrome (Table II)

Weight. Group analysis was not attempted because of the relatively large proportion of trends in the control period. There was a tendency to

decrease weight following estrogen administration in cases 37, 51 and 57. In case 54, there was no significant change, but in case 30 a significant increase occurred.

Water balance. There was no significant group mean difference between periods. There was similarly no significant difference in any case, except in case 30, where the difference of + 260 ml. was significant. A decrease of borderline significance was observed in case 57.

Daily urinary outputs. Cl and Na. Group mean differences between periods were not significant. No significant change was observed in any individual case, except in No. 30 where there was a significant decrease in Cl and Na output following estrogen.

K. Group mean difference between periods was not significant. There was no significant difference between periods in any individual case.

PO_4. This estimation was done in two cases only. A small but significant difference was observed in case 30 ($\bar{d} = -7$ mEq., $P = 0.01$), but there was no significant difference in case 37.

Total serum protein. No significant difference between periods was observed.

Venous hematocrit. There was a highly significant difference in volume of packed cells following estrogen administration, $\bar{d} = -4$ per cent, $P = 0.01$. This represents a mean decrease of 10 per cent below the mean control value.

Plasma Cl and Na. There was no significant difference between periods, $\bar{d} = -0.4$ mEq. per L. Cl, and -2 mEq. per L. Na, $P = 0.8$ and 0.06 respectively.

Thiocyanate space. This observation was made in three cases only, Nos. 37, 51 and 54. There was an increase in the space in each case of between 0.5 and 1.0 L. This increase is of the same order as that found in the normal group.

Figure 4 shows that there was no marked alteration in the rate of gain in average weight during the estrogen period, and that there was no sustained effect on average sodium output, or on water balance. The urinary Na/Cl ratios likewise showed no significant change during the estrogen period.

By calculation, relatively small amounts of Cl, Na and water were apparently retained during the estrogen period, the mean figures being 88 mEq. Cl, 36 mEq. Na, and 330 ml. water. There was a mean increase in weight of 0.6 kg., taken from the last day of the control period. These figures are not in the expected proportion, had the changes been chiefly due to increased ECF volume.

Low plasma protein edema (Table II)

Group analysis was not attempted. In case 69, a small increase of weight during the estrogen period was of borderline significance. This was accompanied by a significant increase in water balance, but no corresponding changes in urinary out-

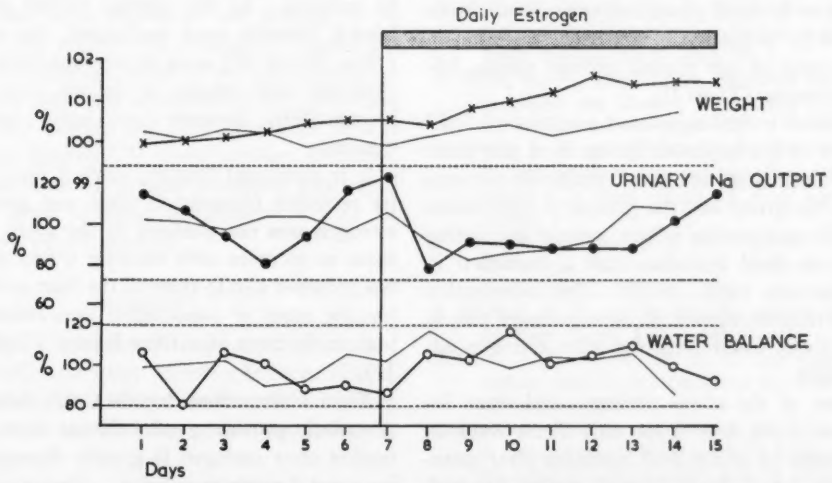

FIG. 4. THE EFFECT OF ESTROGEN ADMINISTRATION ON DAILY WEIGHT, URINARY SODIUM OUTPUT AND WATER BALANCE IN THE NEPHROTIC SYNDROME

See footnote, Figure 1.

put of Cl, Na or K. No significant alterations in the levels of plasma Cl and Na, or of total serum protein.

In case 77, there was a significant increase of weight, but no significant change in water balance, or urinary Cl, Na or K output. Plasma Cl and Na levels were unaltered, but there was a significant increase in total serum protein, associated with an adequate dietary intake while in hospital.

DISCUSSION

In the group of constrictive pericarditis the administration of estrogen was associated with marked and sustained retention of Cl, Na and water (Table I and Figures 1 and 4). Similar results were also observed in the group of cardiac failure, although to a less degree (Table 1 and Figures 2 and 3). Both observations are in significant contrast to the slight and transient effects on NaCl and water metabolism seen in the normal.

In the group of nephrotic cases (Table II, and Figures 3 and 4) the effect of estrogen on NaCl and water metabolism did not differ from the normal as a group. In four of the five individual cases, there was no evidence of NaCl and water retention, but in one case (No. 30) definite retention did occur. This case differed from the remainder in that the nephrotic syndrome had been present for 5 to 6 years as opposed to a maximum of 18 months in the other cases. The NaCl and water retention in this case is unlikely to be due to lowered plasma albumen levels, since there was no indication of abnormal retention in the two cases of low plasma protein edema, following estrogen (Table II).

The failure to find significant retention of NaCl and water in the nephrotic group is of particular interest, since there are two features in common between this group and the groups of constrictive pericarditis and cardiac failure, namely an existing tendency to fluid retention, and a reduction in renal clearance rates (3–6). This observation indicates that the kidney plays no primary role in the NaCl and water retention after estrogen administration.

In view of the above findings, and since involvement of the liver is the only factor common to all groups in which fluid retention after estrogen occurred and absent from all groups in which such fluid retention did not occur (1), it seems likely that the factor deciding this fluid retention is hepatic. Furthermore, it seems reasonable to suppose that this hepatic factor is impaired inactivation of the hormone.

In both constrictive pericarditis and cardiac failure due to other causes, the hepatic circulation is prominently affected. There is increased hepatic vein pressure and sinusoidal congestion (5, 7, 8). Where cardiac output is low in cardiac failure of various etiologies, hepatic blood flow is reduced in proportion (7). Although hepatic blood flow has not been specifically studied in constrictive pericarditis, cardiac output and renal blood flow are reduced (5) and it is reasonable to assume that hepatic blood flow is reduced also.

Hepatic biopsy studies (8, 9) indicate that although hepatocellular necrosis and atrophy may occur in cardiac failure, these changes are never so extensive as to lead to hepatocellular failure (as in portal hepatic cirrhosis). These histological changes closely parallel the clinical severity and duration of the cardiac failure, and are, moreover, reversible, healing taking place when the failure is brought under control.

In our group of constrictive pericarditis, four of the cases were early and asymptomatic, and hepatocellular damage may therefore be presumed minimal. This was in fact demonstrated in two (Nos. 52 and 59) of these four cases by hepatic biopsy. Even in one of the more severe cases (No. 46), structural changes were also found to be minimal. In the cardiac failure group, no hepatic biopsies were performed, but two cases (Nos. 32 and 47) were recovering from first and clinically mild attacks of failure, and in these hepatocellular damage was again presumably minimal.

It is significant that the marked NaCl and water retention observed in these two groups after estrogen was not confined to the more advanced cases or to cases with existing edema or ascites, but occurred also in three of the four early asymptomatic cases of constrictive pericarditis and in both early cases of cardiac failure (Tables I and II).

These observations, together with those already described, provide good evidence that fluid retention after estrogen is greatly dependent upon impaired hepatic circulation. Strong support is therefore provided for the hypothesis that differ-

ing degrees of hepatic circulatory impairment leading to differing rates of estrogen inactivation, may be responsible for the marked fluid retention in cirrhosis with ascites, as contrasted with the slight retention in cirrhosis without ascites, after the administration of estrogen (1).

Furthermore, since our results demonstrate that exogenous estrogen can cause fluid retention in the above groups, it becomes possible that endogenous estrogen may have a similar affect. Increased estrogen activity may, therefore, be a factor in the naturally-occurring fluid retention, not only of constrictive pericarditis, but of any form of cardiac failure where the hepatic circulation is permanently affected.

The above observations do not however, exclude the action of other Na-retaining hormones, and the finding of a sodium-retaining factor resembling aldosterone in the urine of cases of hepatic cirrhosis (10) and cardiac failure (11) suggests that this hormone is also involved.

The actual role of aldosterone in the fluid retention of these conditions remains to be determined. Increased aldosterone excretion appears to be related rather to low urinary Na levels than to the disease process or state of hydration (11). Since control urinary Na levels in our cases were either within the normal range or high, it would appear that fluid retention after estrogen is not dependent on an existing high degree of aldosterone activity. In addition, the fluid-retaining effect of estrogen has been shown in our studies to be closely if not exclusively, related to hepatic involvement by disease, whereas urinary aldosterone is markedly increased in nephrosis and in normal subjects on a low-Na diet (11), where the liver is presumably healthy. It would appear, therefore, on the evidence at present available, that fluid retention after estrogen administration may be independent of aldosterone.

SUMMARY

1. Daily administration of estradiol caused marked NaCl and water retention in cases of constrictive pericarditis, and in cardiac failure of other origin, but not in two cases of peripheral venous obstruction.

2. The same dosage of estradiol caused no significant NaCl and water retention in a group with the nephrotic syndrome, nor in two cases of low plasma albumin edema without renal disease.

3. These findings indicate that the NaCl and water retention following estrogen administration is dependent mainly upon disturbances of the hepatic circulation, and that the kidney plays no primary role.

4. This evidence supports the hypothesis that the quantitative difference observed between the fluid retention of cirrhosis with ascites and that of cirrhosis without ascites following estrogen administration may be accounted for by hepatic circulatory factors.

5. It is further suggested that increased estrogen activity may be a factor in the fluid retention of cases of cardiac failure in which the hepatic circulation is prominently affected.

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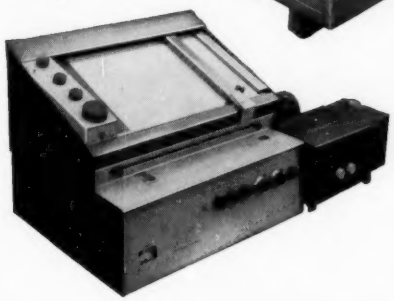

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